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Advances in thrombin generation

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Advances in thrombin generation

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Table of contents

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Editorial: Advances in thrombin generation

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Editorial on the Research Topic
Advances in thrombin generation

The thrombin generation (TG) test is a global coagulation assay (1). An increased TG has been associated with thrombosis (2–4), whereas a decreased TG can result in bleeding episodes (5–9). This collection of articles illustrates the recent clinical and technical advances that have been made in the field of thrombin generation. The articles in this collection describe novel clinical observations using the TG test, the clinical relevance of TG and its role in the personalization of treatment, the development and use of the TG assay in whole blood, and the development of new TG based assays (**Figure 1**).

Whereas TG used to be a typical research laboratory test, the development of fully automated TG analyzers has introduced TG to the clinical laboratory (10). As a result, TG has become of interest to support the personalization of treatment in bleeding and thrombotic diseases (Valke et al.). For example, TG-dependent activated protein C resistance is higher in antiphospholipid syndrome patients compared to controls (Gehlen et al.). A clear difference between patients and controls can be detected (11), although more research is needed to determine the diagnostic and prognostic value of TG in diagnostic strategies. Indeed, previous studies have shown that an imbalance of pro- and anticoagulant processes could be indicative of clinical outcomes in APS (12, 13). Therefore, the availability of the TG assay in the clinical laboratory is an important opportunity for TG-based personalized treatment. Carlo et al. describe the implementation of the thrombin dynamics method on ST Genesia acquired data. The thrombin dynamics method was originally developed for the semi-automated TG assay (Calibrated Automated Thrombinoscopy; CAT). Thrombin dynamics analysis allows a more in-depth analysis of TG curves measured on ST Genesia, bringing us one step closer to TG-based personalized medicine in antiphospholipid syndrome patients and other patient groups.

Clinical observational studies using the TG test have shown that TG remains high in COVID-19 patients that are admitted to the intensive care unit. Hence, increased TG is hypothesized to contribute to the prothrombotic phenotype in COVID-19 patients (van de Berg et al.). Moreover, vaccination against COVID-19 with ChAdOx1-S was associated with a prothrombotic TG profile with a shortened lag time and increased peak height in the weeks after vaccination (de Laat et al.). Furthermore, Feugray et al. show that TG can be used as an indicator for vaso-occlusive crisis in sickle cell disease patients. Additionally, TG analysis in the Moli-sani cohort (14) revealed that increased TG is

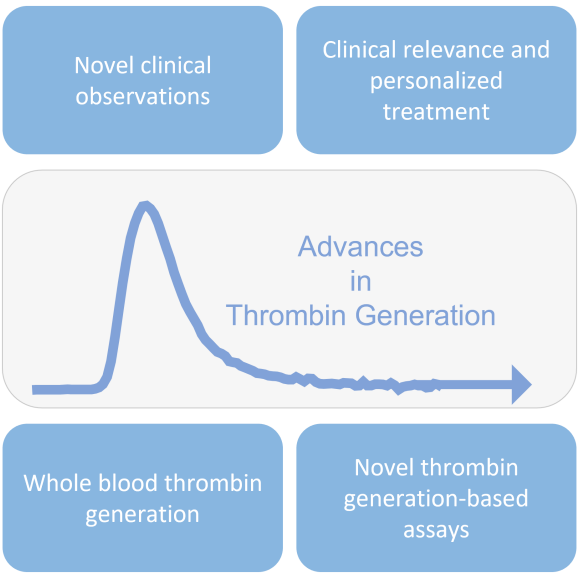

FIGURE 1
An overview of the topics in the articles in the advances in thrombin generation collection.

associated with higher BMI and blood lipid levels with increased TG parameters (de Laat-Kremers et al.). These findings may partly explain the increased risk of cardiovascular diseases in individuals with obesity and/or dyslipidemia.

An important factor in the advancement of TG is the development of novel, specialized TG assay protocols, designed to detect either a specific patient phenotype or to shed more light on mechanistical defects in a patient population of interest. Bai et al. reports the development of specialized TG assays in which the individual role of FII, FV or FX in the TG process can be assessed in an individual. Calibrated Automated Thrombinoscopy (CAT) is the most widely used method for the measurement of TG worldwide (11). Under some circumstances, for example in pediatrics research, it is difficult to obtain the necessary volume of plasma samples to allow the measurement of TG (15). Nevertheless, the growing interest in TG has led to development of the MidiCAT, which uses only half the amount of sample required for the regular CAT (16). Charles et al. have externally validated the MidiCAT and report that the experimental variation for both the regular CAT and midi-CAT were low and that the agreement between MidiCAT and CAT to be satisfactory.

Another TG method that attracts more and more attention is the whole blood TG assay (17). Li et al. shows that multiple myeloma patients have a disbalanced whole blood TG profile, which may explain the paradoxically high prevalence of bleeding and increased risk of thrombo-embolism. Moreover, the whole blood TG assay was used to study the intrinsic coagulation pathway-mediated TG in mice (Konrath et al.), showing an additional advantage for whole blood TG in animal studies.

In conclusion, this collection gives an overview of the advances made in the field of thrombin generation, including but not limited to its usefulness in the clinic as a predictor for personalized therapeutic strategies, the assessment of hemostatic changes in COVID-19 patients and the validation of the MidiCAT for use in studies with small sample volumes. All together, the advances in the field of thrombin generation now allow the incorporation of the TG test in the clinical management of certain patient groups, and novel TG-based assays are expected to follow.

Author contributions

RL-K drafted the manuscript and SZ and BL critically revised it. All authors contributed to the article and approved the submitted version.

Conflict of interest

RL-K and BL are employees of Synapse Research Institute, part of Diagnostica Stago SAS. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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ORIGINAL RESEARCH
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Increased BMI and Blood Lipids Are Associated With a Hypercoagulable State in the Moli-sani Cohort

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The coagulation system can be assessed by the thrombin generation (TG) assay, and increased TG peak height, endogenous thrombin potential (ETP), and velocity index are associated with an increased risk of thrombosis. Obesity had been reported to increase TG and is associated with dyslipidemia, which also predisposes to atherosclerotic cardiovascular disease (CVD). However, the effect of the blood lipid profile on TG has not been studied extensively. To gain more insight into the associations of TG, body mass index (BMI) and lipid profile, we studied TG in relation to these parameters in a large Italian population cohort, the Moli-sani study ($N = 22{,}546$; age $\geq$ 35 years; 48% men). TG was measured in plasma samples collected at the enrollment of subjects in the Moli-sani study. TG was triggered with 1 or 5 pM tissue factor, and TG parameters lag time, peak, ETP, time-to-peak (TTP) and velocity index (VI). Additionally, thrombomodulin was added to assess the function of the activated protein C system during TG. In both women and men, overweight (BMI 25–30 kg/m^2) and obesity (BMI $>$ 30 kg/m^2) were significantly associated with higher ETP, peak and VI (all $p < 0.001$). High total cholesterol, triglycerides and LDL-cholesterol levels were significantly associated with increased ETP and peak (all $p < 0.001$). Linear regression analysis revealed that the ETP is positively associated with both plasma LDL and HDL cholesterol levels, whereas the velocity index is positively associated with HDL cholesterol. Additionally, ETP, peak and VI were significantly associated with the plasma triglycerides content. In conclusion, our study shows significant associations of high BMI and blood lipid levels with increased TG parameters, and this hypercoagulability may partly explain the increased risk of CVD in individuals with obesity and/or dyslipidemia.

Keywords: lipids, BMI, Moli-sani, thrombin, thrombin generation

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INTRODUCTION

Cardiovascular disease (CVD) remains among the most important chronic diseases, contributing to more than 17 million deaths per year globally (1). A major cause of CVD is atherosclerosis, characterized by the formation of a plaque or lesion in artery walls (2). One of the key risk factors for atherosclerotic CVD events is the blood lipid profile: low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) have opposite effects on CVD risk, consistent with the role of LDL and HDL particles in promoting and protecting against atherosclerosis, respectively (3–5). Overweight and obesity [defined as a body mass index (BMI) of more than 25 or more than 30 kg/m^2, respectively (6)] are key risk factors for dyslipidemia (7) and independently associated with an increased risk for arterial and venous thrombotic CVD (8, 9). On the other hand, abnormalities in coagulation and hemostasis represent a well-known link in the relationship between increased BMI and thrombotic risk.

Interestingly, in many individuals with obesity, changes in the hemostatic and fibrinolytic activity are observed that lead to hypercoagulability. For instance, obesity has been associated with elevated levels of tissue factor (TF), coagulation factor (F)VII and FVIII, von Willebrand factor and fibrinogen (10, 11). Furthermore, obese patients present a hypofibrinolytic state owing to higher levels of the plasminogen activator inhibitor 1 (12). Overall, the resulting pro-thrombotic state may contribute to the development of CVD and venous thromboembolism (VTE) in patients with obesity. A promising tool to assess an individuals' coagulation potential is the measurement of the *in vitro* thrombin generation (TG) profile. TG is a global coagulation assay that describes the potential of a blood (13) or plasma (14) sample to generate thrombin and promote coagulation. It allows the quantification of the combined effects of changes in pro- and anticoagulant factors, thus predicting if there is an increased risk of bleeding or thrombosis (15, 16). Indeed, increased TG was found to be associated with first- and recurrent VTE (17, 18), and with myocardial infarction (19).

Obesity has been reported to increase TG in case-control (20) and small cohort studies (21), and weight loss has been shown to reduce TG (22). However, the effect of the blood lipid profile on TG has not been studied extensively. Mechanistically, LDL-C has been reported to support the assembly of the pro-thrombinase complex, and thereby enables the generation of thrombin (23, 24). Moreover, statins have been reported to lower TG, but this antithrombotic effect might be not related to their lipid-lowering properties, at least in part (25). A study by Kim et al. (26) in healthy subjects ($n = 448$) reported significant correlations between several TG parameters and levels of total cholesterol, LDL-C, HDL-C and triglycerides. However, this study did not include clinical data nor medical history, and no data on well-known risk factors for hypercoagulable status-such as obesity–was collected. Therefore, a large cohort study is needed to gain further insights into the associations between TG, lipids and BMI. The Moli-sani cohort consists of 24,325 subjects from the general population of whom detailed information on diet, lifestyle and information on morbidity and mortality was collected. We measured TG profiles in plasma samples from the participants of the Moli-sani cohort, with the aim to investigate the association of BMI and blood lipids content with TG.

METHODS

Study Population

The Moli-sani cohort was randomly recruited in the Molise region (Italy) from city hall registries, as previously described (27, 28). The Moli-sani study complies with the Declaration of Helsinki and was approved by the ethics committee of the Catholic University of Rome, Italy. In total, 24, 325 subjects were enrolled between March 2005 and April 2010. All participants provided written informed consent. For the current study, TG was measured in a total of 22,866 subjects, due to a lack of sufficient plasma volume for the remaining participants (**Figure 1**). Additionally, subjects with incomplete baseline questionnaires were excluded, as well as subjects using anticoagulants, leaving 22,546 observations for statistical analysis. At the time of enrollment in the study, none of the subjects received direct oral anticoagulants.

Blood Collection, Plasma Preparation and Storage

Venous blood samples were previously obtained by venipuncture between 07:00 am and 09:00 am from participants who had fasted overnight and had refrained from smoking for at least 6 h (27). Citrated plasma samples for this study were stored in straws containing the sample code and barcode in liquid nitrogen in a dedicated biobank (http://www.neuromed.it/biobanking-centre/) for 8.2 years (IQR: 7.2 to 9.2 years). Platelet poor plasma was prepared by centrifugating twice at 2,821 g for 10 min. The samples were express-shipped in 3 shipments on dry ice to Synapse Research Institute, Maastricht, the Netherlands on 27-10-2016, 08-05-2017 and 23-06-2017, where they were immediately stored at−80°C. Levels of labile coagulation factors (FV and FVIII) were determined in a subset of 144 randomly chosen samples from the first shipment to confirm plasma sample quality. FV and FVIII levels were measured on the STA-R device using STA deficient FV and STA deficient FVIII reagents, according to the manufacturer's specifications (Diagnostica Stago, Asnières, France). All coagulation factor levels measured were in line with the reference ranges, established by the manufacturer.

Thrombin Generation Measurements

TG was determined in platelet-poor plasma (PPP) using the Calibrated Automated Thrombinography (CAT) method (29). Due to the number of samples in the cohort, every condition was measured in a single well, for each sample. To standardize the measurement procedure as much as possible, commercial reagents from one batch were used to measure TG for all samples. Commercial calibrator, PPP Reagent Low, PPP Reagent [with and without thrombomodulin (TM)] and FluCa kits were purchased from Diagnostica Stago (Asnières, France). All reagents were stored at 4°C, according to the manufacturer's recommendation. Analyses were performed in 96-well round bottom microplates

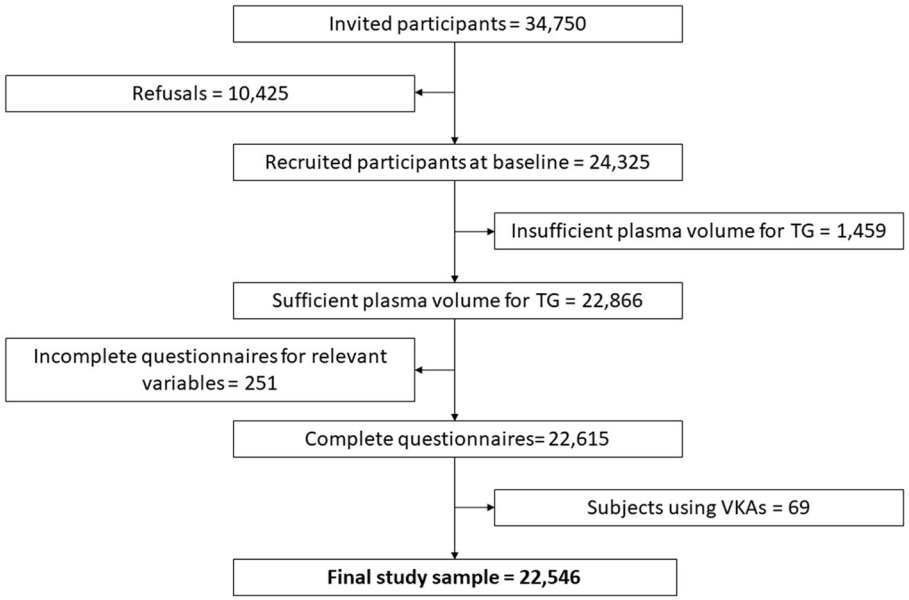

FIGURE 1 | Flowchart of selection of the studied population among Moli-sani participants. The groups of eliminated participants (of the 24 325 recruited at baseline) overlap and the scheme shows sequential exclusion criteria. Therefore, the patients in each eliminated group do not fulfill the previous elimination criteria, but it is possible that patients on VKAs were previously eliminated because of e.g., insufficient plasma volume for thrombin generation.

(Immulon 2 HB plate, Fisher Scientific, Roskilde, Denmark). Each well contained 20 μL PPP Reagent Low, PPP Reagent or calibrator and 80 μL plasma. The samples were preincubated for 10 min and analysis was performed at 37°C in a fluorescence plate reader (Fluoroskan Ascent, Diagnostica Stago, Asnières, France). TG curves and calibration curves were measured immediately after the automatic dispensing of 20 μL FluCa. The TG curve was monitored for 50 min, or shorter if all curves reached the baseline level earlier, to ensure that the TG curves could be calculated in a reliable way. TG curves were measured and analyzed by the Thrombinoscope software (version 5.0), as specified by the manufacturer (Diagnostica Stago, Asnières, France). TG parameters lagtime (time until the first trace of thrombin is formed), peak height (maximum amount of active thrombin present), time-to-peak (TTP, time until the maximum amount of active thrombin is present), area-under-the-curve (endogenous thrombin potential, ETP), and maximum slope of thrombin formation (velocity index, VI) were quantified automatically by the software.

Determination of CVD Risk Factors at Baseline

Blood glucose, serum lipids and C-reactive protein (CRP) were measured within 3 h from blood sampling by enzymatic reaction methods using an automatic analyzer (ILab 350, Milano, Italy). LDL-C level was calculated according to the Friedewald formula (30). Height and weight were measured at baseline, and body mass index (BMI) was calculated as kg/m^2. For specific analyses, BMI was grouped into three categories as normal (<25 kg/m^2), overweight (25–30 kg/m^2) or obese (>30 kg/m^2), according to WHO guidelines (6). A status of "current smoker" was defined as smoking 1 or more cigarettes per day on a regular basis. Waist and hip circumferences were measured according to the WHO guidelines (31) and used to calculate the waist-to-hip (WH) ratio.

Statistical Analyses

The normality of continuous variables was assessed graphically in histograms and normality Q-Q plots (data not shown). Distributions of serum triglycerides, blood glucose, and serum CRP concentrations were skewed and are therefore presented as median and interquartile range (IQR). Values for continuous non-skewed variables are presented as means ± SD. Categorical variables are presented as frequencies. Comparisons of continuous variables between two groups or more than two groups were performed using the Student's *t*-test or Mann-Whitney U test and analysis of variance F test, respectively. Associations between continuous variables were tested using the Spearman's rank correlation coefficient.

The effect of blood lipid levels (total cholesterol, triglycerides, LDL-C, HDL-C) and BMI on TG parameters was evaluated by linear regression analyses. Crude linear regression models stratified by gender were generated with the TG parameters ETP/Peak/LT/TTP/VI as main outcomes and BMI or the different types of lipid parameters (total cholesterol, LDL-C, HDL-C, triglycerides) as exposures. To build the multivariate models, the following strategy was applied: (1) simple univariate regression (UVR) analysis was used to identify independent/predictor variables associated with the dependent/outcome variable (ETP, peak, VI) at the level $P < 0.05$; (2) all the variables identified in the univariate analysis were inserted in a multiple regression (MR) analysis. For all models,

TABLE 1 | General characteristics of the Moli-sani cohort, stratified by sex.

Variable	Women (n = 11,766)	Men (n = 10,780)	P-value
Age, *y*	55.1 ± 11.7	56.1 ± 11.9	<0.001
Current smoker, %	20.4	26.1	<0.001
BMI, *kg/m²*	27.9 ± 5.3	28.2 ± 4.0	<0.001
Waist:hip ratio	0.89 ± 0.09	0.95 ± 0.06	<0.001
Total cholesterol, *mg/dL*	216.5 ± 41.6	210.1 ± 41.8	<0.001
LDL-C, *mg/dL*	131.4 ± 35.4	129.1 ± 34.9	<0.001
HDL-C, *mg/dL*	62.8 ± 14.7	51.9 ± 12.9	<0.001
Triglycerides, *mg/dL*	97 (63)	124 (88)	<0.001
Blood glucose, *mg/dL*	93 (16)	100 (19)	<0.001
CRP, *mg/L*	1.56 (2.36)	1.47 (2.00)	0.002

Data are presented as the mean ± SD or median (IQR) for normally distributed and skewed continuous variables, respectively, and as a percentage for categorical variables. Statistical differences between men and women were analyzed using a Student's t-test or Mann-Whitney U test for normally distributed and skewed continuous variables, respectively, and a Pearson's χ^2 test for categorical variables. BMI, Body Mass Index; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; CRP, C-reactive protein.

TABLE 2 | Thrombin generation (TG) parameters in the Moli-sani cohort, stratified by sex.

TG parameter	Women (n = 11,766)	Men (n = 10,780)	P-value
PPP Reagent low			
ETP, *nM min*	1754.7 ± 419.3	1685.4 ± 400.2	<0.001
Peak, *nM*	370.6 ± 89.9	356.8 ± 86.4	<0.001
Lag time, *min*	3.94 (1)	4 (1)	<0.001
Time-to-peak, *min*	6.00 (1)	6.33 (2)	<0.001
VI, *nM/min*	166.2 ± 64.8	160.4 ± 61.6	<0.001
PPP Reagent			
ETP, *nM min*	1766.9 ± 433.5	1698.4 ± 412.3	<0.001
Peak, *nM*	374.8 ± 83.4	355.7 ± 79.8	<0.001
Lag time, *min*	2.67 (1)	2.67 (1)	<0.001
Time-to-peak, *min*	5.00 (1)	5.00 (1)	<0.001
VI, *nM/min*	167.8 ± 57.3	156.9 ± 54.7	<0.001
PPP Reagent + Thrombomodulin			
ETP inhibition, %	12.24 (9)	12.63 (11)	<0.001
Peak inhibition, %	0.84 (4)	1.83 (5)	<0.001

Data are presented as the mean ± SD or median (IQR) for normally distributed and skewed continuous variables, respectively. PPP, platelet poor plasma; ETP, endogenous thrombin potential; TTP, time to peak; VI, velocity index.

the normality of residuals and homoscedasticity were evaluated by plotting standardized residuals against the predicted values. In case of violation of one or both of these conditions, the variable was not included in the model. The presence of collinearity among independent variables was tested by the variance inflation factor (VIF). A VIF of ≥10 indicates that the variable under consideration is almost a perfect linear combination of the independent variables already in the equation, in which case the variable is not added to the regression equation.

Further associations between TG parameters and general laboratory results and patient characteristics were performed by dividing the TG parameters into quartiles and performing ANOVA analysis on the groups to detect significant differences between high and low parameter values.

A two-sided P-value < 0.05 was considered statistically significant for all analyses. IBM Statistical Package for Social Sciences (SPSS) version 25 software was used for all statistical analyses (SPSS Incorporated, Chicago, USA). Figures were prepared using GraphPad Prism version 5.00 (GraphPad Software, San Diego, USA).

RESULTS

Moli-sani Population

We first determined baseline general characteristics for the subsection of the Moli-sani cohort in which we studied TG (**Table 1**). Our study population consisted of slightly more women (n = 11,766; 52.2%) than men (n = 10,780; 47.8%). On average, male subjects were older, more frequently smoking and had a slightly higher BMI than female subjects. Men had lower total cholesterol, LDL-C as well as HDL-C levels than women, although these differences were small. In contrast, male subjects had substantially higher triglyceride levels than female subjects. Blood glucose was higher in men, whereas CRP levels were slightly higher in women.

TG [as well as the risk for bleeding and thrombosis (32, 33)] is known to be influenced by age (34) and sex (35). **Table 2** presents TG parameters measured in platelet poor plasma with the two types of TF concentrations, stratified by sex. For both the PPP reagent low and PPP reagent, women had significantly higher TG ETP, peak and VI, in combination with a shorter lagtime and TTP, compared to men. The inhibition of the ETP by thrombomodulin (TM) was significantly reduced in women (ETP_{TM-} = 1,764 ± 436 nM·min vs. ETP_{TM+} = 1,426 ± 536 nM·min) compared to men (ETP_{TM-} = 1,692 ± 418 nM·min vs. ETP_{TM+} = 1,346 ± 518 nM·min). This difference was slightly larger when considering only women taking oral contraceptives (n = 934), with an ETP inhibition of 12.11% (IQR 9%) and peak inhibition of 0.66% (IQR 4%), compared to male subjects ($p < 0.001$) (data not shown).

The relationship of the ETP with age was also explored. Both in PPP reagent low and PPP reagent-induced TG, subjects in the lowest quartile of the ETP values were significantly ($p < 0.001$) younger (mean age 54.5 ± 11.1 and 54.4 ± 11.1 years) compared to the individuals in the highest quartile of the ETP values (mean age 57.5 ± 12.7 and 57.4 ± 12.7 years), respectively. Additionally, the inhibition of the ETP by the anticoagulant actions of TM was inversely associated with age: subjects in the lowest quartile of ETP inhibition by TM were significantly older [median age 58 (IQR 21)] than the subjects whose ETP responded more to the inhibitory effect of TM [median age 53 (IQR 16)]. The other TG parameters were not significantly associated with age in the current Moli-sani cohort.

Obesity Is Associated With Increased TG

In both women and men, overweight (BMI 25–30 kg/m^2) and obesity (BMI>30 kg/m^2) were associated with an increased TG,

characterized by increased ETP, peak and VI and prolonged lagtime and TTP (**Supplementary Table 1**). Inhibition of the ETP by the anticoagulant actions of TM was on average lower in obese men compared to men in the other BMI categories, while no significant differences were observed for TM-dependent inhibition in women.

These differences were further confirmed in linear regression models, where higher BMI was significantly associated with higher ETP and VI. The BMI of subjects in the upper quartile of the ETP was on average 8% higher than that of subjects in the lowest ETP quartile ($p < 0.001$). The same trend was observed for VI, where BMI was 4% higher in individuals in the upper quartile of VI distribution, compared to those in the lowest quartile ($p < 0.001$). Finally, a high BMI was associated with a more coagulable state of the blood, as measured by TG.

The Relation Between TG Parameters and Blood Lipid Levels

Since high BMI is related to altered blood lipid composition, we next studied the relationship between lipid profiles and TG in our study population. Blood lipid levels were divided into two groups according to the National Cholesterol Education Program (NCEP) guidelines (36), see **Supplementary Table 2**. Older individuals had significantly higher (≥240 mg/dL and ≥190 mg/dL) total cholesterol and LDL-C levels, respectively. Whereas, subjects with high triglyceride levels (≥200 mg/dL) were more frequently men, the groups with high total cholesterol (≥240 mg/dL), high LDL-C (≥190 mg/dL) and high HDL-C (≥60 mg/dL) contained more women. BMI, proportion of smokers, WH-ratio, CRP and glucose levels were all significantly higher in individuals with lipid levels above the cutoff threshold, except for HDL-C ≥60 mg/dL, which contained on average fewer smokers and more subjects with lower BMI, WH-ratio, CRP and glucose levels. Similarly, TG parameters were generally significantly increased in individuals with lipid levels above the cutoff, except for HDL-C, for which most differences in TG parameters between the two lipid level categories were non-significant (except for the time-dependent parameters).

The relation between lipids and TG was further explored in an association analysis, based on quartiles of the TG parameters ETP and peak (**Table 3**). LDL-C and HDL-C showed different associations with TG: individuals in the lowest quartile for ETP and peak had the lowest plasma LDL-C levels, whereas the HDL-C content did not differ between the quartiles. LDL-C levels were 9% higher in subjects in the highest compared to the lowest quartile of peak TG ($p < 0.001$), and 14% higher in those in the highest compared to the lowest quartile of the ETP ($p < 0.001$).

Finally, linear regression analyses were performed to investigate to what degree lipid levels contribute to the (normally distributed) TG parameters ETP, peak and VI (**Table 3**). In simple UVR, most lipid parameters were significantly associated with each of the TG parameters. Exceptions were HDL-C levels, which were not significantly associated with TG peak height in PPP reagent low-induced TG and with ETP in PPP reagent-induced TG, and LDL-C which was not associated with the VI in PPP reagent-induced TG. Triglyceride levels significantly contributed to PPP reagent low and PPP reagent-induced TG parameters. Total cholesterol had limited added predictive value for TG parameters (also due to collinearity) and was hence not included in most of the final MR models. ETP and peak were mainly affected by triglycerides and LDL-C, whereas VI was also influenced by HDL-C for both TG conditions. Overall, R^2 values of the final models were low, indicating that only a small proportion of the variance in TG parameters could be explained by lipid levels.

The Contribution of BMI, Lipids and Related Factors to TG

Many factors are known to influence TG, besides the blood lipid profile. **Table 1** summarizes the most important variables that affect TG parameters. To adjust for these variables, multiple linear regression analysis was performed for all TG variables to investigate whether, and to what degree the lipid profile influences TG results, adjusting for other differences related to the lipid profile between the subjects (**Table 4**).

Total cholesterol levels are associated with a slightly prolonged lag time and time-to-peak, an elevated ETP and a reduced velocity index (**Table 4**). Triglycerides are positively associated with the lag time and time-to-peak, whereas the ETP and peak height significantly increase with increased triglyceride levels. LDL-C is posivitly associated with a prolonged lag time and time-to-peak, and an increased ETP, whereas HDL-C is associated with a reduced lag time and time-to-peak, and a lower TG peak height.

Additionally, as expected, age, sex and BMI were important influencers of TG parameters, especially, ETP, time-to-peak and velocity index. Smoking was associated with a significantly shorter lag time, and higher levels of acute phase reactant C-reactive protein were associated with a higher TG peak height and velocity index.

DISCUSSION

TG is a promising tool to assess blood coagulability, as it allows quantification of the global hemostatic potential of blood and/or plasma (37). Hence, TG provides information about an individual's hemorrhagic or thrombotic tendency (38–41). Over the past decades, associations between TG and the risk of bleeding or thrombosis have been reported in both prospective and case-control studies. For instance, studies have found an association of increased TG with myocardial infarction (19), coronary artery disease (42) and VTE (15, 17, 43, 44). The increased risk of cardiovascular disease (CVD)-related morbidity and mortality associated with obesity is known to reduce after weight loss (45–48). Other classical risk factors for the increased risk of CVD in individuals with high BMI are systemic inflammation (CRP), dyslipidemia and glucose intolerance (49). However, up to 20% of all coronary events occur in the absence of these risk factors, suggesting that hypercoagulability might play an important role in the relation between BMI and the occurrence of a cardiovascular event (49).

This is, to the best of our knowledge, the first large cohort study investigating the association between BMI and

TABLE 3 | Linear regression and quartile analysis of lipid levels as determinants for TG parametersdetermined using PPP reagent.

		Cholesterol mg/dL)	Triglycerides (mg/dL)	LDL cholesterol (mg/dL)	HDL cholersterol (mg/dL)
Lag time	β-coef. UVR	0.002***	0.001***	0.003***	−0.005***
	β-coef. MR	COL	0.001***	0.003***	- 0.005***
	Q1	203 (52)	97 (67)	121 (43)	58 (20)
	Q2	211 (54)***	107 (73)***	128 (44)***	57 (20)***
	Q3	216 (56)***	115 (79)***	134 (45)***	55 (19)***
	Q4	221 (59)***	127 (90)***	137 (49)***	52 (18)***
ETP	β-coef. UVR	1.97***	0.50***	2.17***	
	β-coef. MR	1.13***	0.51***	0.80**	
	Q1	200 (52)	98 (68)	120 (44)	56 (19)
	Q2	209 (54)***	107 (73)***	126 (43)***	56 (20)
	Q3	215 (53)***	112 (79)***	132 (45)***	56 (19)
	Q4	220 (58)***	117 (82)***	136 (48)***	56 (19)
Peak	β-coef. UVR	0.29***	0.10***	0.27***	0.15***
	β-coef. MR	COL	0.20***	0.22***	0.38***
	Q1	204 (56)	99 (70)	124 (47)	55 (20)
	Q2	208 (53)***	105 (74)**	126 (44)***	56 (19)
	Q3	212 (54)***	111 (74)***	129 (44)***	56 (20)
	Q4	219 (56)***	119 (84)***	134 (47)***	56 (19)
Time-to-peak	β-coef. UVR	0.003***	0.001***	0.004***	−0.006***
	β-coef. MR	COL	0.001***	0.005***	−0.006***
	Q1	202 (50)	101 (70)	119 (42)	57 (20)
	Q2	211 (54)***	107 (74)***	128 (45)***	57 (19)*
	Q3	214 (55)***	111 (77)***	132 (46)***	55 (20)***
	Q4	220 (59)***	120 (88)***	137 (49)***	54 (19)***
Velocity index	β-coef. UVR	0.05***	0.04***		0.06*
	β-coef. MR	0.01	0.06***		0.15**
	Q1	211 (57)	128 (47)	105 (74)	55 (19)
	Q2	210 (55)	128 (45)	104 (73)	56 (20)**
	Q3	209 (54)	127 (45)	108 (75)**	56 (20)*
	Q4	214 (54)***	130 (45)***	117 (82)	56 (19)

*Linear regression results are presented as β-coefficients and their associated p-values. Lipid levels were quantified as the median and IQR for the quartiles of each TG parameter. (Bonferroni corrected) p-values were indicated as *p < 0.05, **p < 0.01, ***p < 0.001. Sex was coded as ''0" for men and ''1" for women, hence a negative β-coefficient indicates a decrease in outcome (TG) variable in women compared to men. Smoking was coded as ''0" for non-smokers and ''1" for current smokers, hence a negative β-coefficient indicates a decrease in outcome (TG) variable in smokers compared to non-smokers. Significant variables shown in the table were included in the final model. β-coef., β-coefficient; ETP, Endogenous thrombin potential; UVR, univariate regression analysis; MR, multiple regression analysis; COL, variable excluded from the model because of collinearity (VIF ≥ 10), Q, quartile.*

hypercoagulability, through TG parameters. Our findings demonstrate that overweight and obesity are associated with higher TG ETP, peak and VI. As these changes reflect hypercoagulability (29), they may thus provide an additional explanation for the increased cardiovascular risk in obese individuals. Our results are in accordance with previous studies showing that obesity is associated with alterations in the coagulation and fibrinolytic system (50). For instance, elevated levels of procoagulant factors FVII, FVIII, and fibrinogen were found in obese patients, which could explain the increased ETP (51, 52). Previously, Ay et al. found that bariatric surgery and consequent weight loss resulted in a significant reduction in TG in morbidly obese adults (22). Extrapolating these findings, it could be hypothesized that underweight (BMI < 18.5 kg/m^2) may be associated with significantly decreased TG parameters. However, in the current study population there were <0.5% ($n = 87$) underweight individuals, hence they were categorized as a "normal" BMI (i.e., up to 25).

Remarkably, the TG lag time was significantly prolonged in overweight and obese individuals compared to individuals with a normal BMI, but only for the PPP reagent low-induced TG. This is surprising, as an increased TG is often accompanied by a shortened lag time (53). The lagtime is predominantly determined by levels of tissue factor pathway inhibitor (TFPI), protein S (PS), factor VII (FVII), FIX and fibrinogen. Interestingly, a correlation between TFPI levels and BMI has been previously reported, demonstrating that an increased obesity index is associated with elevated TFPI levels (54). TFPI downregulates tissue factor-induced TG, which is reflected in a slower onset of TG, hence a prolonged lag time.

TABLE 4 | Determinants for TG parameters in linear regression analysis.

	Lag time		ETP		Peak		Time-to-peak		Velocity index	
	β-coefficient		β-coefficient		β-coefficient		β-coefficient		β-coefficient	
PPP reagent low	**UVR**	**MR**	**UVR**	**MR**	**UVR**	**MR**	**UVR**	**MR**	**UVR**	**MR**
Age, y	0.003***	0.001*	−3.51***	−4.93***			−0.005***	−0.005***	0.47***	0.24***
Sex	0.17***	0.092***	−69.4***	−55.5***	−13.9***	−15.0***	0.168***	0.148***	−5.74***	−9.88***
BMI, kg/m2	0.025***	0.008***	14.0***	12.4***	2.55***	1.46***	0.017***	0.013***	2.55***	0.47***
Smoking	0.038***		−14.6***		−3.19***		0.026*			
Waist:hip ratio	1.191***		111**		68.7***	21.3*	0.334*	−0.645***	65.7***	29.9***
Total cholesterol, mg/dL	0.003***	−0.006***	2.02***	COL	0.34***	COL	0.003***	COL	0.10***	COL
LDL-C, mg/dL	0.004***	0.009***	2.24***	1.87***	0.33***	COL	0.005***	0.005***	0.07***	COL
HDL-C, mg/dL	−0.009***	COL	0.45*	1.79***			−0.009***	−0.007***	−0.09**	−0.49***
TGL, mg/dL	0.002***	0.002***	0.49***	0.63***	0.14***	0.08***	0.001***	0.001**	0.08***	
Blood glucose, mg/dL	0.001***	−0.001***	−0.32**	−0.93***	0.16***		−0.001**	−0.003***	0.18***	0.04*
CRP, mg/L	0.042***	0.033***	20.0***	17.0***	4.17***	3.33***	0.022***	0.018***	2.90***	2.28***
PPP reagent	**UVR**	**MR**	**UVR**	**MR**	**UVR**	**MR**	**UVR**	**MR**	**UVR**	**MR**
Age, y	0.003***	0.001**	−3.41***	−4.77***	−0.19***	−0.47***	−0.003***	−0.003***	0.27***	0.11**
Sex	0.096***	0.047***	−68.5***	−52.8***	19.06***	−19.6***	0.152***	0.129***	−10.9***	−12.8***
BMI, kg/m2	0.017***	0.005***	14.0***	12.4***	2.05***	1.24***	0.016***	0.01***	1.04***	0.34***
Smoking	0.023***	−0.021*	−16.5***		−4.53***		0.026**		−2.14***	
Waist:hip ratio	0.894***	0.132*	93.4**		28.86***	20.2*	0.583***	−0.29**	28.4***	22.6***
Total cholesterol, mg/dL	0.002***	0.003***	1.97***	1.67***	0.29***	COL	0.003***	0.004***	0.05***	−0.02*
LDL-C, mg/dL	0.003***		2.17***	COL	0.27***	COL	0.004***			
HDL-C, mg/dL	−0.005***	−0.006***			0.15***	−0.53***	−0.006***	−0.008***	0.06*	0.16***
TGL, mg/dL	0.001***		0.50***	0.30***	0.10***		0.001***		0.04***	0.05***
Blood glucose, mg/dL	0.001***	−0.001***	−0.34**	−0.96***	0.06**	−0.05*		−0.002***	0.10***	0.04*
CRP, mg/L	0.031***	0.025***	20.2***	17.1***	3.75***	3.24***	0.019***	0.015***	2.49***	2.13***

*Linear regression results are presented as β-coefficients and their associated p-values. P-values were indicated as *p < 0.05, **p < 0.01, ***p < 0.001. Sex was coded as ''0" for men and ''1" for women, hence a negative β-coefficient indicates a decrease in outcome (TG) variable in women compared to men. Smoking was coded as ''0" for non-smokers and ''1" for current smokers, hence a negative β-coefficient indicates a decrease in outcome (TG) variable in smokers compared to non-smokers. Abbreviations: β-coef., β-coefficient; ETP, Endogenous thrombin potential; UVR, univariate regression analysis; MR, multiple regression analysis; COL, variable excluded from the model because of collinearity (VIF ≥ 10); BMI, Body Mass Index; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; CRP, C-reactive protein.*

The effect of TFPI is larger when PPP reagent low. Indeed, we only detected the prolonged lag time in obese patients when PPP reagent low, but not when a higher TF trigger was applied. This is in line with the reports that the PT assay, which uses a high amount of TF, does not show changes in clotting time in obese individuals (55). In addition to elevated TFPI levels in obese individuals, increased fibrinogen levels can contribute to a prolonged lag time as well. Previous reports suggest that initial traces of thrombin formed at the start of the TG measurement can bind to fibrinogen/fibrin, and thereby hinder the feedback activation of upstream coagulation factors by thrombin, and subsequently contribute to a prolongation of the lag time (56).

Regarding the blood lipid profile, we found that TG parameters were significantly higher in individuals with high total cholesterol, triglycerides and LDL-C, and low HDL-C. This is in accordance with the known effects of LDL-C and HDL-C on the risk of CVD: LDL-C is considered to be a risk factor for atherothrombosis, whereas HDL-C is reported to be protective (57), although there is controversy whether the latter applies to certain patient subgroups (58). Triglyceride levels were found to contribute significantly to both PPP reagent low and PPP reagent-induced TG parameters. It has been shown that patients with hypertriglyceridemia had higher FVII and FVIII (59) levels than patients with normal levels of triglycerides. Another study found significant correlations of total cholesterol and triglyceride levels with all procoagulant factors II, VII, IX, and X (26). Of note, in these studies, plasminogen, antithrombin levels (59), protein C and PS (26) were also significantly associated with triglyceridemia. Hence, high triglyceride levels may induce both elevated procoagulant factors and increased anticoagulant factors, suggesting a natural compensatory mechanism for the lipid-associated increase in blood coagulability. From a clinical perspective, the effect of statins in reducing the risk of CVD is known to be not only related to their capacity to normalize hyperlipidemia, but also among others to their antithrombotic effect (60). Indeed, Tripodi et al. (25) previously showed that statins reduce TG in patients with hyperlipidemia. Our data corroborate this association of hyperlipidemia with hypercoagulability, through evidence in a large population cohort.

In conclusion, our study showed significant associations of BMI and blood lipid levels with TG results. These findings aid in interpreting the results of TG in individuals with high lipid levels. Moreover, they underline the importance of hemostatic abnormalities, in addition to metabolic abnormalities, in explaining the increased risk of thrombosis in obese individuals with obesity and/or dyslipidemia. Our results indicate that there is an interplay between the altered blood lipid profile, reported alterations in coagulation factor levels, and TG, even after adjustment with the reported effect of obesity on TG (51, 52). Further studies are needed to confirm the added value of measuring TG to predict the risk of thrombosis, both in healthy individuals and subjects with obesity and dyslipidemia. As clinical follow-up data from the Moli-sani cohort are available, we are currently conducting analyses to determine the relationship between TG and clinical outcome in terms of myocardial infarction, stroke and other thrombotic events.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author. The data are stored in an institutional repository (https://repository.neuromed.it) and access is restricted by the ethical approvals and the legislation of the European Union.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Catholic University of Rome, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RdLK conceived, designed and organized the measurement of thrombin generation in the Moli-sani study, analyzed and interpreted the data, and drafted and revised the manuscript. ADiC analyzed and interpreted the data and revised the manuscript. LvdV analyzed and interpreted the data, and drafted and revised the manuscript. SC managed data collection of the Moli-sani Study and revised the manuscript. MN interpreted the data and revised the manuscript. ADeC managed the Bio-bank of the Moli-sani study. AG revised the manuscript. DH, CB, and DY participated in the data acquisition, interpreted the data, and revised the manuscript. CC participated in the data acquisition and revised the manuscript. GdG and MBD originally inspired the Moli-sani study, promoted the Authors collaboration, and revised the manuscript. BdL conceived, designed and organized the measurement of thrombin generation in the Moli-sani, study, analyzed and interpreted the data, and drafted and revised the manuscript. LI originally conceived and designed the Moli-sani study, promoted the Authors collaboration, interpreted the data, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.897733/full#supplementary-material

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APPENDIX

Moli-sani Study Investigators

The enrolment phase of the Moli-sani Study was conducted at the Research Laboratories of the Catholic University in Campobasso (Italy), the follow up of the Moli-sani cohort is being conducted at the Department of Epidemiology and Prevention of the IRCCS Neuromed, Pozzilli, Italy.

Steering Committee: Licia Iacoviello*°(Chairperson), Giovanni de Gaetano* and Maria Benedetta Donati*.

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Hospitals: Presidi Ospedalieri ASReM: Ospedale A. Cardarelli – Campobasso, Ospedale F. Veneziale – Isernia, Ospedale San Timoteo - Termoli (CB), Ospedale Ss. Rosario - Venafro (IS), Ospedale Vietri – Larino (CB), Ospedale San Francesco Caracciolo - Agnone (IS); Casa di Cura Villa Maria - Campobasso; Ospedale Gemelli Molise - Campobasso; IRCCS Neuromed - Pozzilli (IS).

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Baseline Recruitment staff is available at https://www.moli-sani.org/?page_id=173

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BRIEF RESEARCH REPORT
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Patients With Multiple Myeloma Have a Disbalanced Whole Blood Thrombin Generation Profile

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Background: Multiple myeloma (MM) is associated with a high prevalence of bleeding and an increased risk of thrombo-embolism. MM patients have reduced platelet- and red blood cell (RBC) numbers in blood, which may indicate that the paradoxical hemostasis profile is a consequence of a disturbed platelet and RBC homeostasis.

Objectives: To get better insight in the disbalanced hemostasis of MM patients.

Methods: We conducted a case-control study on the whole blood (WB) coagulation profiles of 21 MM patients and 21 controls. We measured thrombin generation (TG) in WB and platelet poor plasma (PPP) of MM patients and controls.

Results: In WB-TG, we observed that the median time to the thrombin Peak was 52% longer in MM patients than in controls, while the median endogenous thrombin potential until the Peak (ETPp) was 39% higher in MM-patients than in controls. In line with these findings, the levels of platelets, RBCs, white blood cells and agonist induced platelet activation were decreased in MM patients compared to controls. The plasma TG experiments showed no differences between MM-patients and controls.

Conclusion: Patients with MM have a disturbed blood cell metabolism and a disbalanced WB-TG profile. This disbalance may explain the paradoxically high prevalence of bleeding symptoms in MM patients vs. an increased thrombosis risk. There was no disturbance observed in plasma TG, indicating that blood cells are the major determinants for the disbalanced hemostasis in MM patients.

Keywords: blood cells, multiple myeloma, platelet function, thrombin generation, thrombosis

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INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy of the plasma cells, which is characterized by the production of abnormal monoclonal immunoglobulins, called M-protein. Patients with MM have an up to 28-fold increased risk of venous thromboembolism (VTE) and a high prevalence of bleeding complications (1). Although bleeding complications, such as nosebleeds, are common in MM, life-threatening hemorrhage is rare (2). The real cause of the high VTE risk in MM patients remains unclear, despite several

studies on the relation between global coagulation and VTE risk (1, 3, 4). Platelet and red blood cell (RBC) levels of MM patients are reduced, and platelet function is disturbed. This disbalance may lead to both a pro- or an anti-coagulant phenotype, while intensive treatment of MM-patients with anti-platelet therapy, anti-coagulants, chemotherapy, immune modulating drugs (IMiD) and corticosteroids can further modify the thrombosis risk (5).

Common complications in patients with MM are anemia and thrombocytopenia (6, 7). This may disturb the coagulation in MM patients, because activated platelets and RBCs provide the physiological surface for the assembly of coagulation factors and coagulation factor complexes, including tenase and prothrombinase (8–10). Besides high prevalence of thrombocytopenia, platelets of MM patients are more frequently chronically activated during disease progression (11). Pro- or anti-coagulant changes in blood cells may contribute to the increased VTE risk in MM patients and the high prevalence of bleeding complications, and therefore it is important to investigate these cells in combination with coagulation. Therefore, we recently optimized a method to measure thrombin generation (TG) in whole blood (WB-TG) (12). TG is a global coagulation assay that measures the global capacity of blood plasma to form thrombin. Several clinical studies have shown that increased TG in platelet poor plasma (PPP) predicts an increased risk of (recurrent) VTE (13–15). The strength of the new WB-TG assay is that it includes the contribution of platelets, white blood cells and RBCs in the TG measurements (12).

This is the first study that investigates WB-TG coagulation profiles of MM patients and the effects of medication, with regard to a representative control group.

MATERIALS AND METHODS

Study Population

In total, 21 patients with MM were recruited at the Meander Medical Center, Amersfoort, the Netherlands. Patients were included if they were previously diagnosed with MM, were patients at the Meander Medical Center, had a stable condition, and received treatment. Patients were excluded if they were pregnant, had a known coagulation defect, an active infection, bleeding, or other systemic diseases. The control group (n = 21) consists of the partner or a close friend with a comparable age and gender distribution, which donated blood at the same time and location as the patient. Controls were excluded if they had an active malignancy, a personal history of bleeding or thrombosis, were pregnant, had a known coagulation defect, an active infection, bleeding, or other systemic diseases. The study was approved by the Medical Ethical Committee of Maastricht University Medical Center, and patients and volunteers gave full written informed consent according to the Helsinki declaration.

Reagents

Recombinant tissue factor (TF; Innovin®) was purchased from Siemens healthineers, Marburg, Germany. The glycoprotein VI (GPVI) agonist, collagen-related peptide (CRP-XL) was purchased from the University of Cambridge, UK. The fluorogenic substrate Z-Gly-Gly-Arg-aminomethylcoumarin (ZGGR-AMC) was purchased from Bachem (Basel, Switzerland). The calibrator (α2-macroglobulin-thrombin complex) and HEPES buffers containing 5 mg/ml or 60 mg/ml bovine serum albumin (BSA5 and BSA60) were prepared as described by Hemker et al. (16, 17). Synthetic phospholipids (PL) were obtained from Avanti Polar Lipids Inc. (Alabama, USA). MeSADP was purchased from Tocris Biosciences (Bristol, UK), the protease activated receptor (PAR)-1 agonist thrombin receptor activator peptide (TRAP-6 (SFLLRN) was from Bachem (Basel, Switzerland). The monoclonal antibodies used were FITC-conjugated PAC1, directed against the activated αIIbβ3 receptor, PE-conjugated anti-P-selectin (CD62P, clone AK4), APC-conjugated anti-GPIb (CD42b, clone HIP1), APC-conjugated anti-CD14 (monocyte marker) and PE-conjugated anti-αIIbβ3 (CD41a, clone HIP8), all purchased from BD Pharmingen (NJ, USA). HEPES-buffered saline (HBS, 10 mmol/l HEPES, 150 mmol/l NaCl, 1 mmol/l $MgSO_4$, 5 mmol/l KCL, pH 7.4) and fixation solution (137 mmol/l NaCl, 2.7 mmol/l KCl, 1.12 mmol/l NaH_2PO_4, 1.15 mmol/l KH_2PO_4, 10.2 mmol/l Na_2HPO_4, 4 mmol/l EDTA, 0.5% formaldehyde) were prepared.

Blood Collection and Plasma Preparation

Peripheral venous blood from patients and controls was collected aseptically by antecubital puncture via a 21-gauge needle into two 3.2% (109 mM) trisodium citrate vacuum tubes and one K2EDTA (7.2 mg) tube (BD Vacutainer System/Greiner). Citrated blood was used for whole blood experiments and EDTA blood was used for measuring complete blood cell count measured with Sysmex XN-9000 (Sysmex, Germany). All blood samples were kept at room temperature (RT) and used within 4 h after collection. PPP was prepared by double centrifugation of citrated WB at 2,840 g for 10 min. Plasma was aliquoted and frozen at −80°C before analysis.

Whole Blood Thrombin Generation Measurement

WB-TG measurements were performed as described with modifications (12). In short, citrated WB was mixed with the substrate solution (ZGGR-AMC dissolved in BSA60). Subsequently, a solution containing trigger (TF) and $CaCl_2$ was added to the WB and mixed. The volume ratio of WB, substrate solution, and trigger-containing solution was 3:1:2. Of the resulting mixture, 65 μL per well was transferred into 96-well plates (Corning, type number 2595). The final concentrations in the well were 50% WB, 0 or 1 pmol/L TF, 16.7 mmol/L $CaCl_2$, and 416.7 μmol/L ZGGR-AMC. Each blood sample was calibrated by replacing the trigger-containing solution with a calibrator (corresponding with 300 nmol/L thrombin activity). Fluorescence signals were recorded with a Fluoroskan Ascent microplate fluorometer (Thermolabsystems) with λex = 355 nm and λem = 460 nm using Fluoroskan Ascent Software (version 2.6) at 37°C with an interval time of 6 seconds in triplicate. A dedicated preprogrammed spreadsheet template was used to calculate the WB-thrombogram parameters (lag time (minutes), time-to-peak (TTP, minutes), peak thrombin (peak, nmol/L), and

endogenous thrombin potential until the peak (ETPp; nmol x min/L) from the experimental fluorescence data (12).

Platelet Poor Plasma Thrombin Generation Measurement

TG in PPP was measured as described previously, using the calibrated automated thrombinography (CAT) method (18). In short, 80 μl of plasma was added to 20 μl of trigger-solution containing TF and PL. The reaction was started by dispensing 20 μl of substrate solution to the wells. Final concentrations were: 0, 1 or 5 pmol/L TF, 4 μM PL, 16.7 mmol/L CaCl2, and 416.7 μmol/L ZGGR-AMC. Each PPP sample was calibrated by replacing the trigger-solution with a calibrator (corresponding with 600 nmol/L thrombin activity). Data were acquired by specialized software from Thrombinoscope (Maastricht, the Netherlands). Resulting parameters include endogenous thrombin potential (ETP, area under the curve), peak thrombin (peak), lag time (time to thrombin generation initiation) and time-to-peak (TTP, time to reach the peak concentration of thrombin. In each run, normal pooled plasma (NPP) was measured on the same plate. The ETP and peak values of the study subjects were normalized as the percentage of the ETP and peak of the NPP tested in the same run, respectively. The preparation of NPP has been described previously (19). NPP consisted of PPP of 121 apparently healthy individuals (60 males, 61 females) with a median age of 30 (IQR: 25 – 43).

Whole Blood Platelet Activation Test

Platelet activation tests for flow cytometric analysis were prepared as published earlier (20). Test conditions were no agonist, 30 μmol/L TRAP, 0.5 μg/ml CRP-XL, 5 μg/ml CRP-XL and 2 μmol/l MeSADP. Samples were analyzed on an Accuri C6 flow cytometer (BD Biosciences, USA). Median fluorescent intensity (MFI) in the FITC gate and PE gate was selected to determine activated αIIbβ3 (FITC-conjugated PAC1) or P-selectin density (PE-conjugated anti-P-selectin), respectively. The results of the whole blood platelet activation test are missing for 1 patient and 1 control.

Measurement of Platelet Monocyte Complexes

Whole blood (20 μL) was incubated for 15 min with 20 μL reaction mixture consisting of monoclonal antibodies against monocyte CD14 and platelet αIIbβ3. Subsequently, BD FACSTM Lysing solution (Becton-Dickinson, diluted 10-fold with milliQ) was added, vortexed and incubated for 10 min at room temperature before analysis on an Accuri C6 flow cytometer. Gating was performed as described previously (21).

Statistics

Statistical analyses were performed with SPSS version 25 and graphs were generated using GraphPad Prism software version 6. Normality of the data was assessed using the Shapiro-Wilk test. Data are represented as median with interquartile range (IQR; 25–75%). Comparisons between independent groups were performed with the Mann-Whitney U test. The Spearman test was used for correlation analysis. A two-sided P-value < 0.05 was considered statistically significant. Based on the following assumptions: alpha is 0.05, power is 0.80 (beta = 0.20) and an increase in ETPp of 20% in patients compared to controls (mean control group: 321 nmol*min/L; mean MM patients: 385 nmol*min/L; standard deviation: 67.9 nmol*min/L) (12), we calculated that we needed at least 18 patients and 18 controls for our study.

RESULTS

Baseline Characteristics

Baseline characteristics of patients and controls are summarized in **Table 1**. We included 21 MM patients and 21 controls in this study with a comparable age and sex distribution. White blood cell count, RBC count, platelet count, hematocrit and hemoglobin level were decreased, and the immature reticulocyte fraction (IFR), red cell distribution width (RDW) and mean platelet volume (MPV) were increased in MM patients compared to controls. In total, 11 patients and 1 control had anemia (hemoglobin lower than 8.7 mmol/L for men and 7.6 mmol/L for women) and 6 patients were thrombocytopenic (platelet count lower than $100*10^9$/L). M protein level was lower than 10 g/L for most patients (19 of 21), and lower than 15 g/L in all MM patients.

Thrombin Generation Profile in MM

To study the effect of MM on coagulation and specifically determine the contribution of blood cells, we measured thrombin generation in PPP (**Table 2**) and in whole blood (**Table 2** and **Figure 1**) of MM patients and controls. In total, 4 MM patients received anti-coagulant therapy (1 patient received vitamin K antagonist, 1 patient received low molecular weight heparin and two patients received Xarelto) and 2 controls (both received vitamin K antagonists).

The global plasma coagulation profile did not differ between MM patients and controls (**Table 2**). None of the TG parameters were different between patients and controls, indicating that coagulation of MM patients was not affected by a factor in plasma.

The coagulation profile in WB was different between MM patients and controls on several aspects (**Table 2** and **Figure 1**). Patients with MM had a longer lag time and TTP, upon stimulation with 0 pM or 1 pM TF. The median lag time was increased up to 38% and the TTP was up to 52% increased in patients compared to controls. The WB-TG ETPp was higher in MM patients than in controls when TG was stimulated with 1 pM TF, the median ETPp was 39% higher in patients compared to controls. Remarkably, the peak amount of thrombin formed was comparable between MM patients and controls. To exclude that the observed difference between MM patients and controls was caused by the use of anticoagulants, we also performed the analysis in the absence of patients and controls on anticoagulants (see **Supplementary Table 1**). The difference in WB-TG parameters between patients and controls remained.

Effect of Medication on WB-TG Profile

Patients were classified in groups according to their medication usage (anticoagulants, platelet inhibitor, IMiDs, corticosteroid

TABLE 1 | Baseline characteristics of patients and controls.

	All ($n = 42$)	Patient ($n = 21$)	Control ($n = 21$)	*P*-values
Age, years	67 (53–74)	66 (57–75)	67 (51–73)	ns
Female [*n* (%)]	18 (43%)	8 (38%)	10 (48%)	ns
Cell count				
White blood cell count, $*10^9$/L	5.8 (4.2–7.2)	4.5 (4.0–6.9)	6.2 (5.3–7.5)	0.03
Red blood cell count, $*10^{12}$/L	4.5 (3.9–5)	4.0 (3.4–4.5)	4.8 (4.4–5.4)	0.001
Hemoglobin, mmol/L	8.8 (7.9–9.4)	8.1 (6.7–9.1)	9.0 (8.4–9.5)	0.014
Haematocrit, %	41.9 (38.7–44.9)	39.6 (32.6–44.3)	43 (41–46.3)	0.014
Immature reticulocyte fraction, %	12.3 (9.7–17.1)	15.9 (12.9–26.7)	10.1 (8.3–11.6)	<0.001
Red cell distribution width, fL	46 (42.6–55.0)	54.9 (50.3–60.3)	42.6 (40.3–44.8)	<0.001
Mean platelet volume, fL	10.1 (9.6–11)	10.3 (9.8–11.4)	9.8 (9.5–10.5)	0.041
Platelet count, $*10^9$/L	222 (156–285)	178 (76–222)	250 (222–292)	<0.001
Platelet distribution width, fL	11.2 (10.2–12.7)	11.6 (10–13.6)	10.7 (10.1–11.9)	ns
Anemia [*n* (%)]	12 (28.6%)	11 (52.4%)	1 (4.8%)	
Thrombocytopenia [*n* (%)]	6 (14.3%)	6 (28.6%)	0 (0%)	
Medication *[n (%)]*				
Platelet inhibitor	13 (31%)	11 (52.4%)	2 (9.5%)	
Anticoagulants	6 (14.3%)	4 (19%)	2 (9.5%)	
Immunomodulatory imide drugs	14 (33.3%)	14 (66.7%)	0	
Corticosteroid	11 (26.2%)	11 (52.4%)	0	
Proteasome inhibitor	5 (11.9%)	5 (23.8%)	0	
Antibiotics	11 (26.2%)	11 (52.4%)	0	
Laxation	8 (19%)	8 (38.1%)	0	
Gastric acid inhibitor	9 (21.4%)	9 (42.9%)	0	
Ca+Vit D3	5 (11.9%)	4 (19%)	1 (4.8%)	
Antivirus	7 (16.7%)	7 (33.3%)	0	
Antihypertension	10 (23.8%)	7 (33.3%)	3 (14.3%)	
Asthma medication	4 (9.5%)	4 (19%)	0	
Uric acid inhibitor	5 (11.9%)	5 (23.8%)	0	
Cholesterol lowering drug	6 (14.3%)	4 (19%)	2 (9.5%)	

Median and Interquartile ranges (25–75%) or percentage (%) are indicated. Mann-Whitney U test was used for comparison.

or proteasome inhibitor) (see **Supplementary Table 2**). The lag time and ETPp of patients, regardless of the medication used, remained increased in patients with MM compared to controls. Only the use of anticoagulants significantly impacted WB-TG, for the other drugs the WB-TG parameters were not significantly different between patients that did or did not receive a certain drug.

Contribution of Blood Cells to WB-TG

Results from WB-TG showed that blood cells are important contributors to the coagulation profile in MM patients. Therefore, platelet function was studied by measuring the platelet response to different activation triggers (5 μg/mL CRP-XL, 30 μmol/L TRAP and 2 μmol/L MeSADP) using flow cytometry in 20 MM patients and 20 controls (**Table 2**). Irrespective of the agonist used, αIIbβ3 activation was lower in MM patients than in controls. P-selectin expression in response to TRAP was also lower in patients compared to controls, while the MeSADP and CRP-XL induced P-selectin expression did not differ between the two groups. The baseline platelet activation level and the platelet-monocyte complexes (PMC) were comparable between MM patients and controls.

To gain insights into the relation between blood cell fractions and WB-TG in MM patients and controls, we tested the association between WB-TG parameters and the blood cell parameters and platelet activation markers (**Figure 2**). The lag time and TTP were negatively correlated with RBC count, hemoglobin, hematocrit, lymphocyte count and platelet activation makers and positively correlated with RBC indices (RDW and IFR). Peak and ETPp correlated less with blood cell parameters and did not correlate with platelet activation markers.

DISCUSSION

The current study shows that patients with MM have an imbalanced hemostasis. On one hand the ETPp in whole blood is increased, which indicates a hypercoagulable state, while on the other hand the lag time and the TTP in WB-TG are prolonged. This imbalanced hemostasis may explain the paradox that MM patients have a high incidence of venous thromboembolism and a

TABLE 2 | Plasma thrombin generation profiles, whole blood generation profiles and whole blood platelet activation profiles of the study subjects.

	Patients	Controls	*p*-value
Plasma thrombin generation	*N* = 21	*N* = 21	
Lag time (min)			
0 pM TF	16 (13–19)	16 (15–22)	0.237
1 pM TF	6 (5–8)	5 (5–7)	0.84
5 pM TF	3 (3–4)	3 (3–3)	0.504
TTP (min)			
0 pM TF	18 (16–23)	20 (18–25)	0.385
1 pM TF	10 (9–12)	11 (9–12)	0.399
5 pM TF	6 (6–8)	7 (6–8)	0.268
Peak (% of NPP)			
0 pM TF	146 (107–225)	154 (98–183)	0.554
1 pM TF	193 (127–268)	161 (132–204)	0.252
5 pM TF	126 (95–159)	108 (87–125)	0.187
ETPp (% of NPP)			
0 pM TF	140 (105–171)	129 (114–148)	0.554
1 pM TF	151 (120–187)	140 (131–159)	0.642
5 pM TF	112 (94–136)	108 (94–126)	0.87
Whole blood thrombin generation	N = 21	N = 21	
Lag time (min)			
0 pM TF	24 (20–33)	18 (16–22)	0.005
1 pM TF	5 (4–8)	4 (3–5)	0.001
TTP (min)			
0 pM TF	33 (25–42)	24 (21–28)	0.003
1 pM TF	14 (11–19)	9 (8–10)	<0.001
Peak (nmol/L)			
0 pM TF	233 (121–269)	199 (160–278)	0.66
1 pM TF	197 (128–264)	211 (168–266)	0.473
*ETPp (nmol * min/L)*			
0 pM TF	754 (558–854)	577 (492–725)	0.038
1 pM TF	835 (670–1002)	600 (481–732)	0.004
Whole blood platelet activation	*N* = 20	*N* = 20	
αIIbβ3 activation (MFI)			
Baseline	105 (92–149)	115 (104–138)	0.192
TRAP	151 (107–262)	467 (272–561)	<0.001
MeSADP	1,363 (986–1,963)	2,254 (1,354–3,239)	0.018
CRP-XL	3,253 (2,510–4,069)	4,429 (3,329–4,840)	0.008
P-selectin expression (MFI)			
Baseline	90 (84–112)	89 (86–113)	0.883
TRAP	5,842 (5,434–7,014)	7,124 (6,306–7,897)	0.03
MeSADP	2,314 (1,190–2,586)	2,383 (1,052–5,071)	0.512
CRP-XL	6,321 (5,480–7,261)	6,554 (6,085–8,060)	0.242
Platelet-monocyte complexes (%)	6.55% (5.1–11.675%)	6.25% (4.25–9.125%)	0.273

Median and Interquartile ranges (25–75%) are indicated. The Mann-Whitney U test was used to compare between groups. CRP, collagen related peptide; ETPp, endogenous thrombin potential until the peak; MFI, Median fluorescent intensity; NPP, normal pooled plasma; TF, tissue factor; TRAP, thrombin receptor activating peptide; TTP, time to peak.

high prevalence of bleeding symptoms (1, 2, 22, 23). Additionally, we observed lower platelet numbers, impaired platelet function and lower RBC count in MM-patients, which may further explain the high prevalence of bleeding complications in MM. It is our hypothesis that platelets and RBCs play a crucial role in the balance between a pro- and anti-coagulant phenotype in MM patients.

The pro-coagulant phenotype of MM patients may depend on patient- and disease-related factors and on (side-)effects of therapy (24). We did not find a strong effect of medication on WB-TG profiles, although the numbers of patients in this sub-group analysis does not allow to draw firm conclusions.

Previous studies observed increased plasma levels of FVIII and VWF, increased activated protein C (APC) resistance and

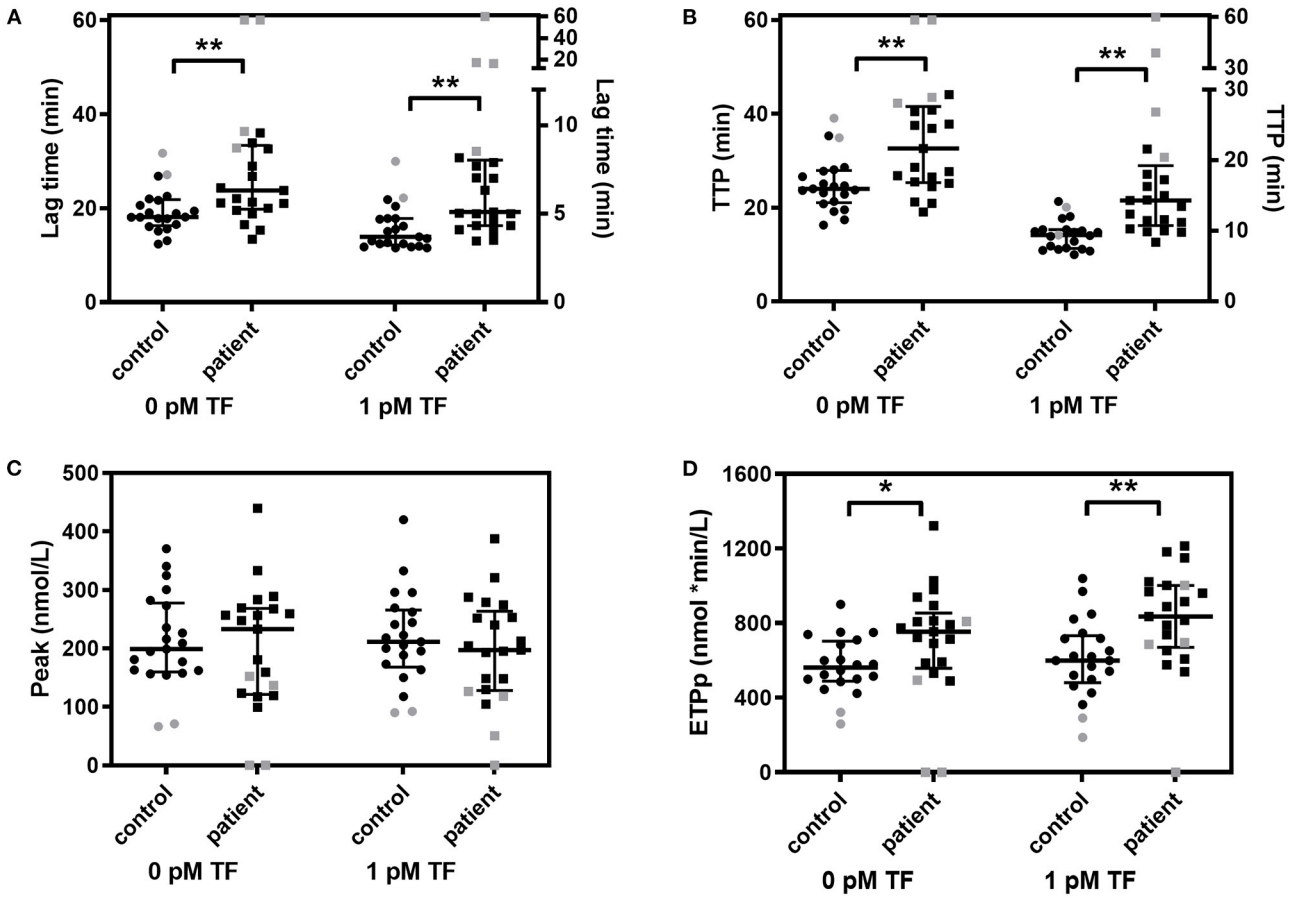

FIGURE 1 | Whole blood thrombin generation in multiple myeloma (MM) patients and controls. Thrombin generation was stimulated with 0 pM and 1 pM TF in whole blood of MM patients and controls. **(A)** Lag time, **(B)** peak thrombin, **(C)** time-to-peak (TTP) and **(D)** endogenous thrombin potential until the peak (ETPp) are presented. Median and IQR are presented as error bars. Patients and controls on anticoagulant medication are indicated in gray. $^{*}P < 0.05$, $^{**}P < 0.01$.

reduced soluble thrombomodulin levels in MM patients (25). Furthermore, patients with MM have more phosphatidylserine (PS) positive blood cells (26) and hyperactivation of platelets (11, 27). Our study confirms that blood cells, especially RBCs and platelets, are crucial players in the disbalanced hemostasis in MM. We showed that TG in the absence of blood cells (PPP) was comparable between patients and controls, whereas in WB the TG parameters were indicative for both a hypo- and a hypercoagulable state. Furthermore, MM patients had a significant lower platelet and RBC counts than controls and the response of platelets to platelet agonists was reduced. We showed that platelet function, RBC numbers, RBC turnover and RBC distribution were associated with WB-TG parameters, indicating that platelets and red blood cells regulate the coagulation phenotype. We did not find a relation between white blood cells and thrombosis phenotype in MM-patients, although we think that this should be further studied with more dedicated TG-tests. Remarkably, our findings that WB-TG lag time was prolonged in MM patients, suggest that TF on microparticles or on white blood cells, as was previously observed (28), does not explain our findings of an imbalanced coagulation profile in MM patients.

The recent improvements of the WB-TG approach is a major strength of our study, which allowed us to explore coagulation in MM-patients in the presence of blood cells (12). Several studies have tried to study coagulation in MM-patients with standard plasma thrombin generation profiles. The results of those studies were inconclusive: some studies observed an increased TG (ETP) in PPP (29–32), other studies, including ours, found no difference between MM patients and controls (33, 34) while there are also studies that reported a reduced TG (ETP) in MM patients (35, 36). One study observed no effect on the ETP and Peak, however, the TTP was reduced and the velocity index increased in MM patients compared to controls (37). Altogether, there is no consistent evidence that plasma of MM patients was pro-coagulant, implying that the pro-coagulant phenotype of MM-patients may be a consequence of the blood cell components.

In line with our observations, another study investigating the role of autologous platelets in TG found that MM patients are pro-coagulant (32). Using PRP-TG, it was shown that ETP was higher in MM patients than in controls, while Lenalidomide treatment further increased the ETP in PRP-TG. The lag time nor TTP were reported in this study (32). Our observation that ETPp

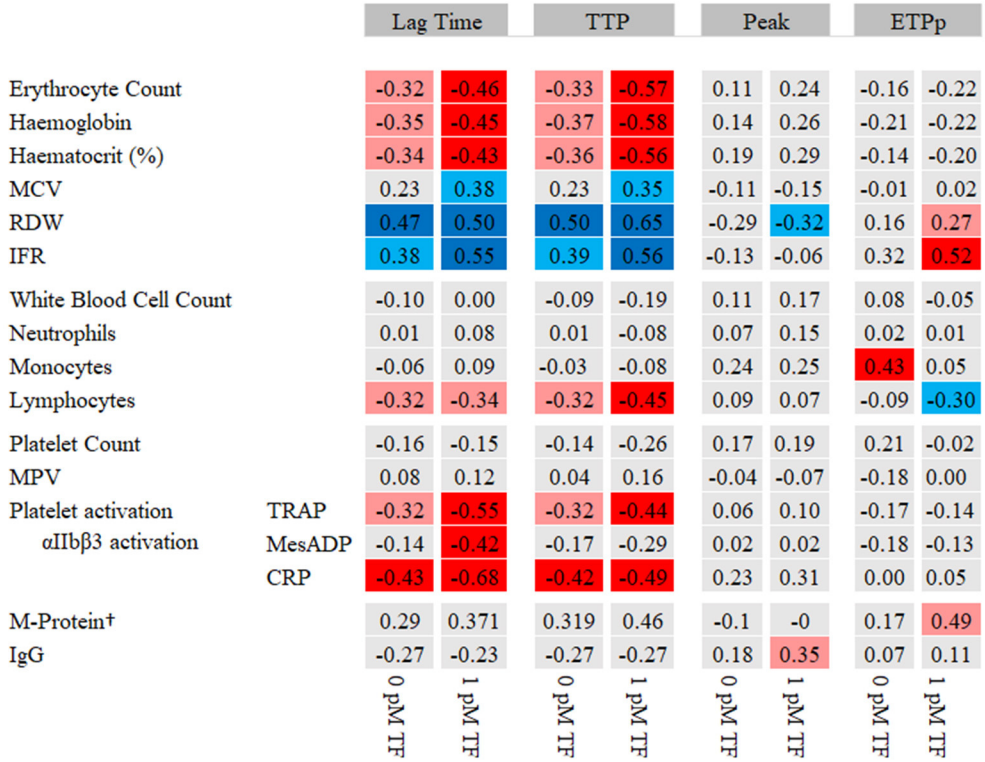

FIGURE 2 | Heat map of the correlation between WB-TG parameters and blood cell parameters. Spearman correlation statistics are shown. In red, a negative correlation with lag time and TTP and a positive correlation with peak and ETPp are presented. In blue, a positive correlation with lag time and TTP and a negative correlation with peak and ETPp are presented. $P < 0.05$ depicted in light red/light blue. $P < 0.01$ depicted in dark red/dark blue. †M-protein levels were only determined in patients. CRP, collagen related peptide; ETPp, endogenous thrombin potential until the peak; IFR, immature reticulocyte fraction; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MPV, mean platelet volume; RDW; red cell distribution width; TRAP, thrombin receptor activating peptide; TTP, time to peak.

in MM patients was increased in WB-TG confirms this finding. Furthermore, we observed that the lag time and the TTP in a WB-TG setting were prolonged in MM-patients. This fragile balance between anti- and pro- coagulant phenotype is in line with the clinical knowledge that MM patients have both a high bleeding risk and a high thrombosis incidence (2, 24, 38).

Our findings that 29% of the MM patients were thrombocytopenic, while most of the patients had a reduced agonist induced platelet response, a prolonged lag time and an increased TTP, is in line with clinical knowledge that MM patients have an increased incidence of hemorrhage (38). Platelet dysfunction problems have been previously reported in patients with MM (39) and in patients with hematologic cancers (40). Moreover, it has been suggested that M protein produced by the malignant plasma cells may also directly affect coagulation. In our study, even though all patients received adequate treatment to stabilize M protein levels below 15 g/L, M-protein levels were still positively correlated with ETPp in WB-TG stimulated with 1 pM TF.

RBCs are the most abundant cells in blood, accounting for approximately 35–45% of the blood volume. The current observation that RBC count and high RBC turnover indices were associated with a shorter lag time and TTP is in line with the notion that RBCs promote thrombin generation *in vitro* (10, 41). This was further confirmed by experiments on RBC reconstitution to healthy plasma, which showed that thrombin generation profiles are dose-dependently dependent on RBC count (10, 12). Furthermore, it has been shown that RBC distribution width is associated with higher risk of venous thrombosis and arterial thromboembolism (42, 43), which supports our findings of a correlation between the red cell distribution width and ETPp and peak height in WB-TG.

A major strength of the current study is the inclusion of partners, or close friends of the MM patients as control group, because this ensured comparable distribution for gender, age, ethnic background, lifestyle and social and economic state. Furthermore, patients and their relatives were invited together, to ensure identical pre-analytical treatment of blood at the same time, in the same room, by the same technician.

The most important limitation of our study is the small sample size. Although our study has sufficient power to show that WB-TG parameters were different in MM-patients and controls, our population size does not allow in-depth subgroup analysis to link WB-TG to treatment strategy. Another limitation

is the case control design, which cannot be used to predict disease outcome in MM patients, because we do not have follow-up data.

In summary, we have shown that MM patients have a slower onset of thrombin formation in whole blood, while they have an increased thrombin formation capacity. MM patients seem to balance on a thin line between a pro- and an anti-coagulant phenotype and only minimal triggers can push patients to either thrombotic complications or to bleeding events. The TG in PPP was not different from controls, indicating that the blood cells, but not the plasma, is responsible for the disbalanced hemostatic phenotype of MM-patients. Disbalanced hemostasis may be an effect of disease characteristics, but it may also be a side effect of the intensive treatment of patients. The next logical step will be a large cohort study, which determines the WB coagulation phenotype at baseline to follow the patients on bleeding and VTE incidence during follow up.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committee of Maastricht University Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JK and MR designed the research. RF and KS recruited the patients. LL, MR, and YS performed experiments and collected the data. YS, MR, JW, and JK performed data analysis. LL, MR, and JK wrote the original manuscript. LL, MR, YS, JR, RF, KS, DH, JW, BL, and JK revised the manuscript. JK, MR, and JR supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.919495/full#supplementary-material

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

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

Objectives: To obtain reference values for thrombin dynamics (TD) analysis in 112 healthy donors using an automated system for TG.

Methods: TG was measured on the ST Genesia, fibrinogen on the Start, anti-thrombin (AT) on the STA R Max and α_2Macroglobulin (α_2M) with an in-house chromogenic assay.

Results: TG was measured using STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen. The TG data was used as an input for TD analysis, in combination with plasma levels of AT, α_2M and fibrinogen that were 113% (108–118%), 2.6 μM (2.2 μM–3.1 μM) and 2.9 g/L (2.6–3.2 g/L), respectively. The maximum rate of the prothrombinase complex (PCmax) and the total amount of prothrombin converted (PCtot) increased with increasing tissue factor (TF) concentration. PC_{tot} increased from 902 to 988 nM, whereas PC_{max} increased from 172 to 508 nM/min. Thrombin (T)-AT and T-α_2M complexes also increased with increasing TF concentration (i.e., from 860 to 955 nM and from 28 to 33 nm, respectively). PC_{tot}, T-AT and T-α_2M complex formation were strongly inhibited by addition of thrombomodulin (−44%, −43%, and −48%, respectively), whereas PC_{max} was affected less (−24%). PC_{tot}, PC_{max}, T-AT, and T-α_2M were higher in women using oral contraceptives (OC) compared to men/women without OC, and inhibition by thrombomodulin was also significantly less in women on OC ($p < 0.05$).

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

KEYWORDS

thrombin generation, thrombin dynamics, antithrombin, fibrinogen, oral contraceptives

Introduction

The haemostatic balance is an equilibrium of pro- and anticoagulant factors that work synergistically to maintain haemostasis and prevent bleeding and thrombosis. When coagulation is triggered, both intrinsic and extrinsic coagulation pathways come together in one common pathway that finally leads to the generation of thrombin (1). Thrombin is the key player of the coagulation cascade as it not only cleaves fibrinogen into fibrin monomers, but also evokes the activation of coagulation factors (F) V, VIII, and XI, and the protease activated receptors that are present on platelets (2, 3).

As thrombin has various roles in the regulation of the coagulation cascade, it is desirable to measure thrombin generation (TG) in order to detect possible bleeding or thrombotic phenotypes. Thrombin generation measured with the semi-automated Calibrated Automated Thrombinography (CAT) method has been shown to be associated with the risk of bleeding and thrombosis in clinical studies (4–10). It is a sensitive tool to identify congenital or acquired haemostatic disorders, and can be used to investigate the efficacy of drugs that affect the formation of thrombin [3, 6]. Nowadays, the ST Genesia performs a fully automated TG measurement in platelet poor plasma (11–13).

Several years ago, the thrombin dynamics (TD) analysis was developed for the CAT method (14). This computational method allows the researcher to study the pro- and anticoagulant processes that determine TG: prothrombin conversion and thrombin inactivation. Over the past years, the TD method has shown its usefulness in multiple clinical settings (15–23). TD analysis can pinpoint changes in prothrombin conversion or thrombin inactivation that provoke a change in coagulation. In liver cirrhosis patients, TD analysis provided evidence that indeed TG is rebalanced and that the prothrombin conversion and thrombin inactivation are both reduced due to lower coagulation factor production (19). Moreover, as the TD method partially relies on the computational modeling of the inactivation of thrombin, *in silico* experimentation can be used to predict the effect of changes in prothrombin conversion and thrombin inactivation on the TG curve. *In silico* modeling was used in several clinical settings, for example to study the effect of prothrombin complex concentrate administration with and without added antithrombin (AT) after cardiopulmonary bypass surgery (17), to predict the effect of AT expression targeting in hemophilia patients and investigate inter-patients variability (24), and in trauma patients (25).

As TD analysis has been shown to be clinically relevant in a research setting, we set out to perform TD analysis as previously described,(14) using TG curves acquired on the newly developed ST Genesia (Diagnostica Stago, Asnières-sur-Seine, France) (26). The ST Genesia is a benchtop, fully automated TG assay, related to the previous CAT assay, which is available in clinical laboratories. The use of the ST Genesia for TG has the advantage that it is a more standardized method and has specific reagent kits for specific experimental aims. Differences between the CAT and ST Genesia method, is that the latter one includes the presence of a reference plasma and quality controls in the reagent kits and has a different calibration method (13). We determined reference values for all ST Genesia acquired TD parameters using all currently available ST Genesia reagents (STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen) in 112 healthy donors. These reference ranges can be used for comparison by other research groups after verification in healthy volunteers according to the CLSI guidelines (27). Moreover, we investigated the effect of sex and oral contraceptive (OC) use on TD parameters.

Materials and methods

Blood collection

The population described in this study was previously investigated in a study by Ninivaggi et al. (13). In total, 112 healthy donors were included in this study. The study was conducted according to the Declaration of Helsinki (2013) and approved by the Medical Ethical Committee of the Maastricht University Medical Center. Non-inclusion criteria for this study were the use of drugs interfering with coagulation, having a known coagulation disorder and/or being younger than 18 or older than 65 years. Blood samples were taken from the donors only after signing the informed consent. Vacuum blood drawing tubes (Greiner Bio-One) containing 3.2% sodium citrate (in

a 9:1 ratio blood:citrate) were used to draw the blood from the antecubital vein. To reduce the effect of pre-analytical factors on TG, the tourniquet was only used for the purpose of finding the vein, but loosened afterwards, and the first tube of blood was discarded. Platelet poor plasma was obtained by centrifuging the blood twice for 10 min at 2630 g at room temperature immediately after blood drawing. The anonymized plasma samples were stored directly at −80°C until further use.

Thrombin generation

The ST Genesia was used to measure TG as previously described (13). In short, first a calibration curve was performed by using the STG-Cal&Fluo kit. Once the calibration has been performed successfully, the reference plasma and quality controls were measured. For this study three reagent kits were used that are commercially available: STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen. Each reagent kit contains its own reference plasma that is used to normalize the sample TG data, as well as 2 or 3 quality controls. The STG-ThromboScreen reagent kit contains two activators with the same TF and phospholipid content, but one of them also contains TM purified from rabbit lung. After successful completion of these runs, the plasma samples of the healthy donors were thawed in a warm water bath at 37°C for 5 min. Immediately hereafter, the plasma tubes were gently mixed, placed on board and TG was measured. The samples were run in duplicate and the ST Genesia embedded software analyzed the data automatically and gave a mean result for the duplicates. The reference plasma run in parallel to the samples, comes with specific assigned ranges provided on a barcoded flier and helps to normalize results (26, 28). The ST Genesia normalizes the results for each sample automatically by applying the following formula: [patient sample result / reference plasma result · activity assigned for the particular lot and parameter of this reference plasma].

Plasma levels of antithrombin, fibrinogen, and α_2Macroglobulin

AT, fibrinogen and α_2Macroglobulin (α_2M) levels were measured to perform TD analysis. AT was measured on the automated coagulation analyzer STA-R Max according to manufacturer specifications using STA®-Stachrom ATIII kit (reagent and analyzer Diagnostica Stago, Asnières-sur-Seine, France). Functional fibrinogen levels were measured using the Clauss method with STA®-Liquid Fib on STart (reagent and semi-automated analyzer Diagnostica Stago, Asnières-sur-Seine, France). Plasma α_2M levels were measured with an in-house chromogenic assay as previously described by Kremers et al. (14).

Thrombin dynamics

The TG curve is the net result of prothrombin conversion and subsequent thrombin inactivation (14). The course of prothrombin conversion can be calculated if the thrombin concentration at a certain time point (i.e., a point on the TG curve) and the rate of thrombin inactivation are known. The inactivation of thrombin is a relatively simple process, in which only a few mediators have a clinically meaningful effect. During TG, thrombin is inactivated mainly by AT, and a smaller fraction is inhibited by α_2M and a group of various minor inhibitors. Additionally, the fibrinogen content of a plasma sample has been shown to influence the rate of thrombin inhibition, because fibrin(ogen) binds and thereby protects thrombin from inhibition by its natural inhibitors (14, 29).

Computation of thrombin inactivation

Several years ago, we developed a computational model that predicts the rate of inactivation of a certain amount of thrombin based on the plasma concentrations of AT, α_2M and fibrinogen (14, 17, 18, 30). This model consists of a set of ordinary differential equations, which describe the rate of thrombin inactivation in time based on the plasma AT, α_2M and fibrinogen level and the concentration of free thrombin at each point in time (Equations 1–3).

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

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

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

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

Computation of prothrombin conversion

As previously described, this computational model can be used to extract prothrombin conversion curves from TG data (23). At any moment during the course of the TG process, the thrombin concentration (i.e., the TG curve) is the net result of prothrombin conversion and thrombin inactivation. As a result, the course of prothrombin conversion (d(P)/dt) can be calculated from the TG curve ([T]t) if the inactivation rate of thrombin for a specific thrombin concentration (d(T-inh)/dt) is known (Equation 4). We can calculate the thrombin inactivation rate at each time point during TG, using the model for thrombin inactivation as described above (Equation 5), and the

thrombin concentration obtained from the thrombin generation curve. Additionally, experimentally determined plasma levels of AT, α_2M and fibrinogen are required as an input for the computational model.

$$d(T)/dt = -d(P)/dt - d(T - inh)/dt \quad (4)$$

$$-d(P)/dt = d(T)/dt + k_{AT} \cdot [AT]_t \cdot [T]_t + k_{\alpha 2M} \cdot [\alpha_2 M]_t \cdot [T]_t \quad (5)$$

Similar to the TG curve, the prothrombin conversion curve is typically quantified by several parameters: PC_{tot} (total amount of prothrombin converted during the TG test; area-under-the-curve), PC_{max} (maximum rate of prothrombin conversion, peak height of the curve), T-AT (amount of thrombin-antithrombin complexes formed), and T-α_2M (amount of thrombin-α_2M complexes formed) (14, 23). Additionally, the total inhibitory potential of each plasma is quantified by the thrombin decay constant (TDC), independent of prothrombin conversion, and solely based on the levels of AT, α2M, and fibrinogen (23). The thrombin decay capacity is the pseudo-first order decay constant for thrombin that combines the overall effect of thrombin inactivation by AT and α_2M, and the interference of fibrinogen in this process.

High throughput thrombin dynamics analysis

Thrombin dynamics analysis was performed in Matlab (R2016a, Mathworks, Eindhoven, the Netherlands) using the Matlab "Statistics and Machine Learning Tool Box" (version 10.2, Mathworks, Eindhoven), and theMatlab "Curve Fitting Toolbox" (version 3.5.3, Mathworks, Eindhoven, the Netherlands) and to ensure a high throughput computation of thrombin dynamics parameters. Analyses were performed on a HP probook (HP, Amstelveen, the Netherlands) equipped with a Microsoft Windows 10 pro operating system. The equations described in the sections above were implemented in an semi-automized data analysis script, which analyzes around 50 curves per minute.

Statistics

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

Results

Thrombin generation measured on the ST Genesia

TG was measured using the three reagent kits available for the ST Genesia: STG-BleedScreen ($N = 112$), STG-ThromboScreen ($N = 112$) and STG-DrugScreen ($N = 111$). The TF concentrations of the aforementioned reagent kits are manufacturer proprietary information, however, they range from low to medium and high, respectively. Table 1 summarizes the following TG parameters: lag time, peak height, time-to-peak, Endogenous Thrombin Potential (ETP), velocity index for all reagent kits, and ETP inhibition induced by TM for STG-ThromboScreen kit. As expected, peak height and velocity index increased with increasing TF concentration, while lag time and time-to-peak shorted with increasing TF concentration. The effect on ETP was less pronounced compared to the other parameters. Addition of TM affected especially peak height and ETP, and to a minor extent velocity index, lag time and time-to-peak. The median (IQR) ETP inhibition was 47% (34.9–64.3%).

Thrombin dynamics on ST Genesia TG data

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

The TD parameters PC_{tot}, PC_{max}, T-AT, and T-α_2M are shown in Figure 1. PC_{tot}, increased slightly with increasing TF concentration from 902 nM to 933 nM to 988 nM with the STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen, respectively (Figure 1A). On the contrary, PC_{max} increased strongly with increasing TF concentration and ranged from 172 to 206 nM/min to 508 nM/min for the aforementioned reagent kits (Figure 1C). Similarly to the ETP, T-AT, and T-α_2M complexes increased with the TF concentration (Figures 1E,G). T-AT was, respectively for the STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen, 860, 897, and 955 nM, while T-α_2M was 28, 30, and 33 nM. The effect of TM was measured using the STG-ThromboScreen kit, which as previously described contains TG trigger with and without TM. PC_{tot}, T-AT, and T-α_2M complex formation are strongly inhibited by the addition of TM (−44, −43, and −48%,

TABLE 1 Thrombin generation data.

	STG-BleedScreen		STG-ThromboScreen		STG-DrugScreen	
	Median	IQR	Median	IQR	Median	IQR
Lagtime (min)	2.9	2.6–3.4	2.3	2.1–2.6	1.3	1.2–1.5
Peak (nM)	168	140–204	195	169–238	347	300–405
Time-to-peak (min)	6.4	5.8–7.3	5.4	4.6–6.2	2.8	2.5–3.3
ETP (nM*min)	1112	934–1,325	1,127	993–1,388	1213	1,087–1,419
Velocity Index (nM/min)	60	45–81	84	63–115	328	231–417

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

respectively, Figures 1B,F,H), whereas PC_{max} was affected less, but still significantly (−24%, Figure 1D).

The reference values for each TD parameter and STG reagent were calculated as the 2.5th and 97.5th percentile of the distribution using the data from all 112 healthy donors and are shown in Table 2.

Effect of sex and oral contraceptive use

It has been previously reported that TG and TD parameters (23) differ between men and women, and that the use of OC can influence TG in women (11, 13, 31). Here, we compared three groups: men, women without the use of OC and women taking OC. No significant differences in TG parameters between men and women without OC were found whatever the trigger reagent, except for the lag time, that was consistently shorter in women (Supplementary Table 1). On the contrary, women taking OC showed not only a shorter lag time and time-to-peak, but also a higher peak height and ETP, compared to women that did not use OC or men. A more pronounced consequence of OC usage could be observed after addition of TM as ETP inhibition in women using OC shows a median value of 25.5% only (22.3–32.3%) compared to 57.6% (43.4– 65.8%; $p < 0.0001$) and 45.0% (34.9– 63.3%; $p = 0.0006$) in men and women without OC, respectively.

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

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

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

Discussion

Since the development of the semi-automatic CAT method for the measurement of thrombin generation, computational modeling of thrombin generation, fibrinolysis or more widely the computational modeling of coagulation has been a topic of interest (32–34). Such computational models can vary from complete computational models that generate a thrombin generation curve based on plasma coagulation factor levels and the reaction constants for each reaction in the coagulation cascade (33, 35, 36), to reduced models that only describe a part of the thrombin generation process (37),

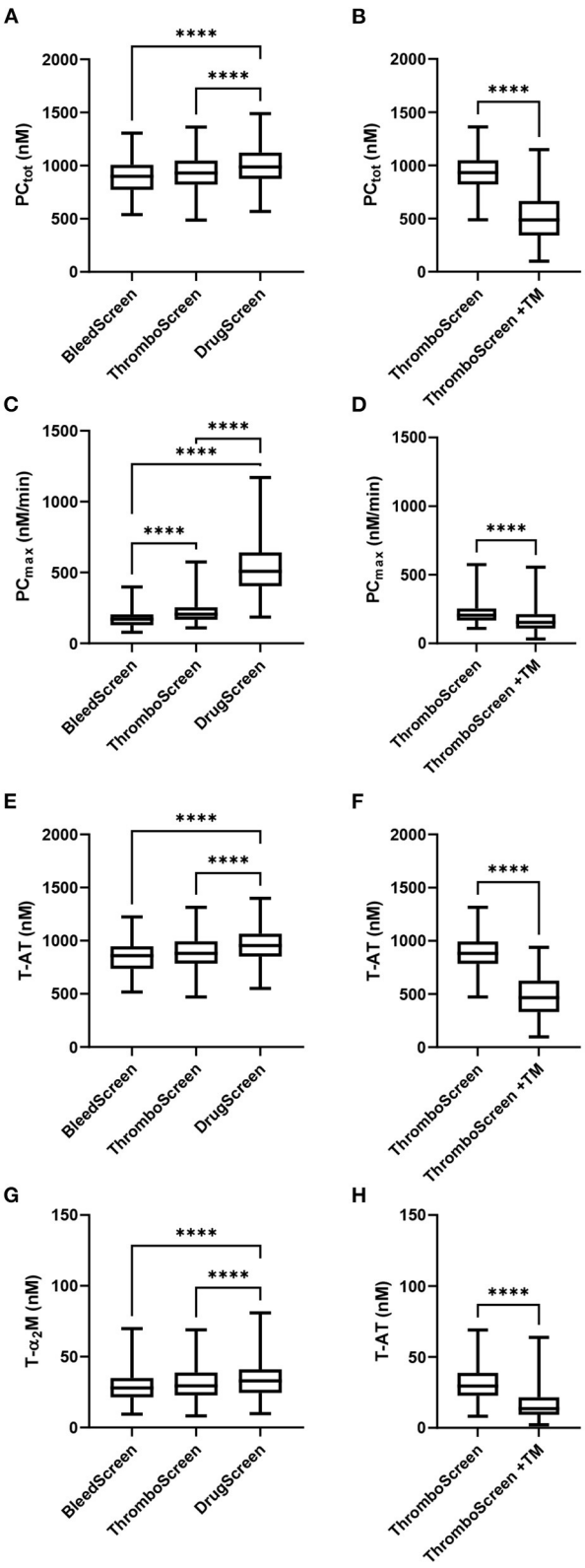

FIGURE 1
Thrombin dynamics parameters obtained from thrombin generation data measured with the ST Genesia. TD analysis was performed on the TG data measured with the STG-BleedScreen($N = 112$), STG-DrugScreen ($N = 111$), and STG-ThromboScreen with and without TM ($N = 112$). TD parameters PCtot **(A,B)**, PCmax **(C,D)**, T-AT **(E,F)**, and T-α_2M **(G,H)** complexes are depicted. Data are median with interquartile ranges and minimum and maximum values. tot, total; max, maximum; TM, thrombomodulin; T-AT, thrombin-anti-thrombin complex; T-α_2M, thrombin-α_2M complex. ****$P \leq 0.0001$.

TABLE 2 Reference ranges for thrombin dynamics parameters determined on the ST Genesia.

	Median	2.5th percentile	97.5th percentile
STG-BleedScreen			
PC_{tot} (nM)	902	609	1239
PC_{max} (nM/min)	172	90	364
TAT (nM)	860	586	1,182
Ta_2M (nM)	28	12	60
STG-DrugScreen			
PC_{tot} (nM)	988	654	1,416
PC_{max} (nM/min)	508	232	1,088
TAT (nM)	955	630	1,364
Ta_2M (nM)	33	17	59
STG-ThromboScreen			
PC_{tot} (nM)	933	542	1,251
PC_{max} (nM/min)	206	115	472
TAT (nM)	897	526	1,202
Ta_2M (nM)	30	15	56
STG-ThromboScreen +TM			
PC_{tot} (nM)	492	188	1,001
PC_{max} (nM/min)	153	56	466
TAT (nM)	470	184	870
Ta_2M (nM)	14	4	41

TG was measured with STG-BleedScreen, STG-DrugScreen, and STG-ThromboScreen on the ST Genesia in 112 healthy subjects and reference ranges were calculated as the 2.5th and 97.5th percentile of the distribution.

such as thrombin dynamics analysis. Thrombin dynamics analysis is a hybrid method that uses both the experimentally generated thrombin generation curve, as a computational model to predict the thrombin inactivation rate to compute the prothrombin conversion curve (14). Therefore, TD analysis pinpoints out changes in the hemostatic balance to the pro- or anticoagulant pathway during TG (14, 23). Several studies using TD analysis based on TG data generated by the semi-automated CAT method have shown its clinical relevance (15–22, 30, 38–40).

In healthy individuals, prothrombin conversion parameters PC_{tot} and PC_{max}, and anticoagulant parameter TDC, are known to be higher in subjects with a non-O blood group,

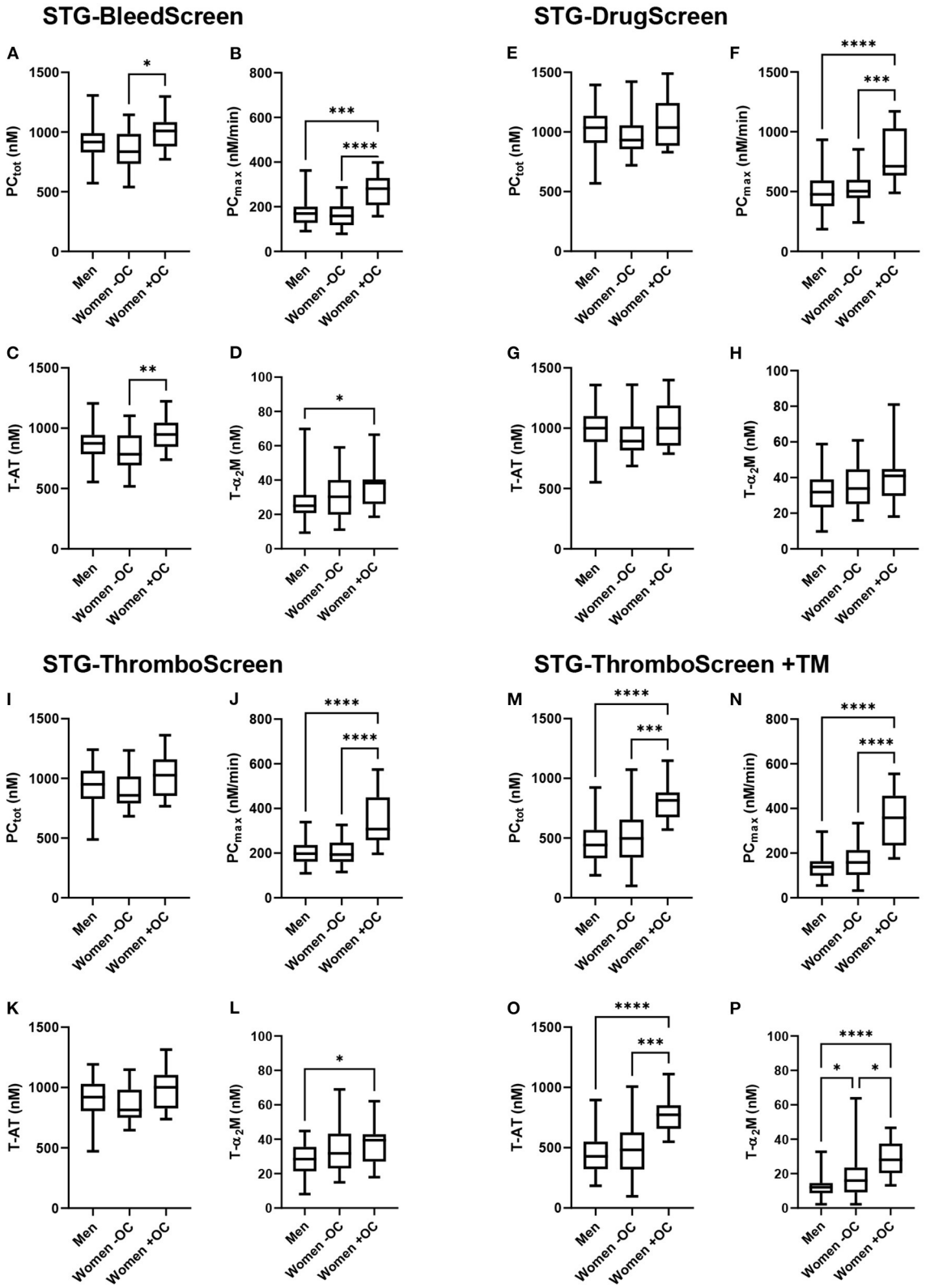

FIGURE 2
Thrombin dynamics parameters obtained from thrombin generation measured with the ST Genesia stratified for sex and oral contraceptive use. TG was measured with the STG-BleedScreen **(A–D)**. STG-DrugScreen **(E–H)**, STG-ThromboScreen without TM **(I–L)**, and STG-ThromboScreen with TM **(M–P)**. TD parameters PCtot **(A,E,I,M)**, PCmax **(B,F,J,N)**, T-AT **(C,G,K,O),** and T-α_2M **(D,H,L,P)** complexes are depicted for each group: men, women without OC and women with OC. Data are median with IQR and minimum and maximum values. tot, total; max, maximum; T-AT, thrombin-antithrombin complex; T-α_2M, thrombin-α_2M complex; TM, thrombomodulin. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

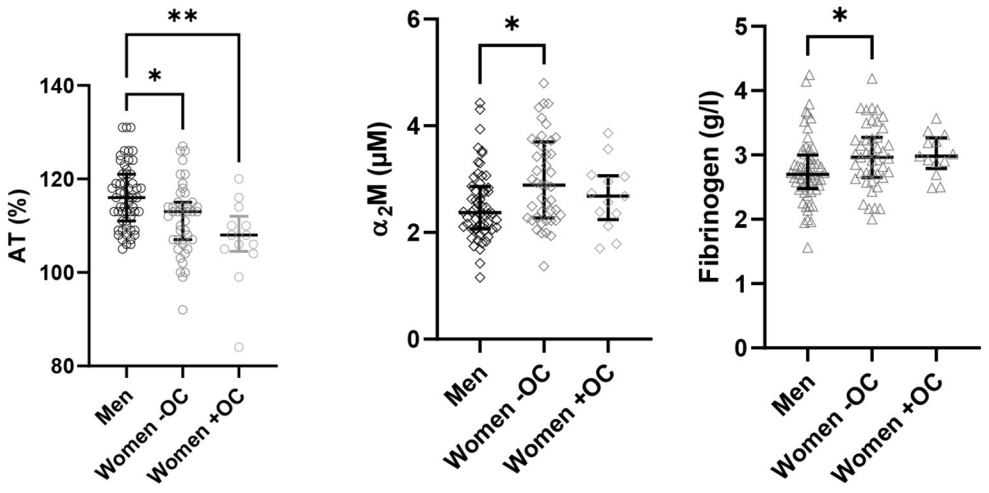

FIGURE 3
Antithrombin, α_2M and fibrinogen levels. The coagulation factor levels were measured in the plasma of 112 healthy donors and are depicted for the three groups: men, women without OC and women with OC. Data are median with IQR. AT, antithrombin; OC, oral contraceptives. *$P \leq 0.05$, **$P \leq 0.01$.

compared to subjects with blood group O (30). Another study pointed out that strenuous exercise reduces prothrombin conversion in healthy subjects (16), and a study in platelet rich plasma showed that platelets have a significant and positive effect on PC_{tot}, PC_{max}, T-AT, and T-α2M complex formation (40). Moreover, thrombin dynamics parameters are known to differ in children compared to adults (18). Prothrombin conversion is known to be higher and T-α2M is more in important in children than adults in the balance between T-AT and T-α2M. Furthermore, in the antiphospholipid syndrome, the hemostatic balance is shifted toward a more pro-thrombotic phenotype due to elevated prothrombin conversion but unchanged thrombin inactivation rates (21). Additionally, within the group of APS patients, increased TG and prothrombin conversion were associated with a history of thrombosis (29).

We set out to perform TD analysis using TG curves acquired on the ST Genesia, which is currently available in clinical laboratories. We determined the reference values for all TD parameters using the ST Genesia reagents (STG-BleedScreen, STG-ThromboScreen, and STG-DrugScreen) in a population of 112 healthy donors. The reference ranges should be verified by other laboratories according to the CLSI guidelines in 120 healthy volunteers, if not feasible, it may be acceptable if local measurements on 20 or more healthy subjects yield comparable results (27, 41). Moreover, we investigated the effect of sex and the use of oral contraceptive (OC) on TD parameters.

An additional advantage of TD analysis is that it offers the possibility to perform *in silico* modeling of the changes in TG when plasma coagulation factor levels change. For example, in the past, TD analysis in cirrhosis patients showed that although the PC_{tot} is reduced due to reduced plasma prothrombin levels, PC_{max} is elevated. However, AT levels are lower in cirrhosis patients due to reduced AT production, which results in a rebalanced TG. Furthermore, *in silico* analysis, that was performed as part of thrombin dynamics analysis, pointed out that the choice of prothrombin complex concentrates containing AT are probably safer for treatment of bleeding in liver cirrhosis patients than prothrombin complex concentrates without AT (19). Indeed, this was confirmed by a clinical study performed by the group of Lisman et al. (42), who found that treatment with prothrombin complex concentrates without AT increased TG by 2-4-fold, whereas the administration of fresh frozen plasma, containing both pro- and anticoagulants, increased TG only slightly. Schöchl et al. (43) demonstrated the importance of AT inclusion in prothrombin complex concentrates for treatment in traumatic and surgical coagulopathy. Additionally, the *in silico* prediction of the increase of TG by silencing miRNA targeting AT expression in individual hemophilia patients is expected to improve the dose targeting of the drug (23, 24).

Since the measurement of TG has been fully automatized on the ST Genesia analyzer, TG curves can now also be acquired in clinical laboratories (12, 13, 44–47). Therefore, we investigated TD analysis using ST Genesia TG data to establish normal ranges for TD parameters in an apparently healthy population. The reference values for TD parameters obtained from ST Genesia TG curves are very comparable to the referenced values previously determined for TD parameters

obtained from CAT data (23). As mentioned previously, the ST Genesia is based on its precursor the CAT assay. They both use the same thrombin-sensitive substrate, but they differ in reagent content and calibration method. TD parameters determined with the STG-BleedScreen reagent, which contains the lowest amount of TF, correspond well-to TD parameters measured with the PPP Reagent Low used in the CAT method (23). Similarly, TD parameters measured with the STG-ThromboScreen without TM and STG-DrugScreen reagent, containing, respectively an intermediate and a high TF concentration, corresponded well-to TD parameter values obtained with the CAT PPP Reagent and PPP Reagent high, respectively (23).

We also investigated the effect of sex and OC use on TD parameters, as these are known to affect TG (13, 48) and coagulation tests in general (49, 50). In our previous study we already demonstrated differences in women taking OC compared to men and women without OC (13). This is in line with the findings in this study, were we demonstrated that TD parameters did not differ between men and women without OC. The only difference found in TD parameters between men and women is the higher amount of T-a_2M complexes found in women compared to men. In CAT TD we found similar results, including a (non-significant) trend toward higher T-a_2M formation in women (23). This discrepancy between men and women can be attributed to the higher level of α_2M in women compared to men, which stimulates the inhibition of thrombin by α_2M (23, 51, 52).

On the contrary, the use of OC has distinctive effects on TD parameters, including the attenuation of the inhibitory effect of TM, as known previously from literature (53, 54). The use of OC causes a significant increase in almost all TD parameters, both in ST Genesia- and CAT-based TD analysis, in the absence of TM (23). The use of OC increases TG through the stimulation of both the total amount and the maximal rate of prothrombin conversion (PC_{tot} and PC_{max}). This is in line with the fact that OC use has an attenuating effect on the activated protein C pathway, which is a natural anticoagulant system that inhibits the production of thrombin (48–50). Although this effect is more pronounced in the presence of TM, it was also observed in its absence. We have previously shown that plasma prothrombin and FX levels are important influencers of prothrombin conversion, as both PC_{tot} and PC_{max} increase dose-dependently with the prothrombin and FX level (23). Remarkably, the effect of FV levels on PC_{tot} and PC_{max} was marginal around its physiological plasma concentration, indicating that OC use might have additional effects on prothrombin conversion besides the well-known inhibitory effect on the activated protein C pathway (48–50). This could also explain why the effect is also detected in the absence of TM.

In conclusion, we showed that TG curves measured on the fully automated ST Genesia device can be used as an input for TD analysis. The data obtained can be used as reference values for future clinical studies (i.e., after verification of these reference values) as the balance between prothrombin conversion and thrombin inactivation has shown to be useful in several clinical settings. Therefore, the introduction of the ST Genesia to the clinic is a future opportunity to also use the TD method in specified clinical settings.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The study was conducted according to the Declaration of Helsinki (2013) and approved by the Medical Ethical Committee of the Maastricht University Medical Centre. Written informed consent was obtained from all participants for their participation in this study.

Author contributions

AC and BD designed the study. RD collected the samples. MN and QY performed the experiments. MN and RD analyzed the data and wrote the first draft of the manuscript. AC, HT, and BD critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AC is full-time employee of Diagnostica Stago S.A.S. QY, MN, RD, and BD are employees of Synapse Research Institute, part of Diagnostica Stago S.A.S.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Population-wide persistent hemostatic changes after vaccination with ChAdOx1-S

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Various vaccines were developed to reduce the spread of the Severe Acute Respiratory Syndrome Cov-2 (SARS-CoV-2) virus. Quickly after the start of vaccination, reports emerged that anti-SARS-CoV-2 vaccines, including ChAdOx1-S, could be associated with an increased risk of thrombosis. We investigated the hemostatic changes after ChAdOx1-S vaccination in 631 health care workers. Blood samples were collected 32 days on average after the second ChAdOx1-S vaccination, to evaluate hemostatic markers such as D-dimer, fibrinogen, α2-macroglobulin, FVIII and thrombin generation. Endothelial function was assessed by measuring Von Willebrand Factor (VWF) and active VWF. IL-6 and IL-10 were measured to study the activation of the immune system. Additionally, SARS-CoV-2 anti-nucleoside and anti-spike protein antibody titers were determined. Prothrombin and fibrinogen levels were significantly reduced after vaccination (−7.5% and −16.9%, $p < 0.0001$). Significantly more vaccinated subjects were outside the normal range compared to controls for prothrombin (42.1% vs. 26.4%, $p = 0.026$) and antithrombin (23.9% vs. 3.6%, $p = 0.0010$). Thrombin generation indicated a more procoagulant profile, characterized by a significantly shortened lag time (−11.3%, $p < 0.0001$) and time-to-peak (−13.0% and $p < 0.0001$) and an increased peak height (32.6%, $p = 0.0015$) in vaccinated subjects compared to unvaccinated controls. Increased VWF (+39.5%, $p < 0.0001$) and active VWF levels (+24.1 %, $p < 0.0001$) pointed toward endothelial activation, and IL-10 levels were significantly increased (9.29 pg/mL vs. 2.43 pg/mL, $p = 0.032$). The persistent increase of IL-10 indicates that the immune system remains active after ChAdOx1-S vaccination. This could trigger a pathophysiological

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mechanism causing an increased thrombin generation profile and vascular endothelial activation, which could subsequently result in and increased risk of thrombotic events.

KEYWORDS

ChAdOx1-S, COVID-19, thrombin generation, vaccination, hemostasis

Introduction

At the end of 2019, the Severe Acute Respiratory Syndrome-Cov-2 (SARS-CoV-2) virus caused a pandemic that has been keeping the world in its grip ever since. By December 2021, more than 260 million people were infected with the virus and over 5.3 million people died as a result of the SARS-CoV-2 virus (1, 2). Several vaccines were developed to reduce the spread of the SARS-CoV-2 virus, including the ChAdOx1-S vaccine, formerly known as "COVID-19 Vaccine AstraZeneca" (3, 4). The ChAdOx1-S vaccine leads to the synthesis of specific SARS-CoV-2-proteins to elicit an immune response against the spike protein of SARS-CoV-2. The ChAdOx1-S vaccine uses a modified chimpanzee-derived adenovirus encoding for the spike-glycoprotein ChAdOx1-S of SARS-CoV-2 (5). Since January 2021, the ChAdOx1-S vaccine has been administered worldwide but predominantly in the UK (6). A study by Jeong et al. (7) reported a SARS-CoV-2 antibody positivity rate of 98.2–100% after two vaccinations, and the overall estimated vaccine efficacy was 74.0% (3). Quickly after the start of vaccination, reports started to emerge that anti-SARS-CoV-2 vaccines, including ChAdOx1-S, could be associated with an increased risk of thrombotic events (5).

In April 2021, European Medicines Agency reported a possible association between ChAdOx1-S and other SARS-CoV-2 vaccines, and a rare syndrome of thrombosis alongside thrombocytopenia, which was named vaccine-induced thrombotic thrombocytopenia (VITT) (8). VITT presents as thrombosis in atypical sites, thrombocytopenia and the presence of autoantibodies against platelet-factor 4 (PF4) (9). The autoantibody against PF4 binds to the platelet FcRγIIA receptor and causes platelets to aggregate.

As vaccination with ChAdOx1-S as well as with Ad26.COV2.S has been linked not only to VITT, but also to other venous and arterial thrombotic episodes without thrombocytopenia (10), we were interested whether vaccination with ChAdOx1-S would be associated with more global hemostatic changes post-vaccination. Therefore, we studied a large range of hemostatic parameters in a cohort of health care workers in a Belgian tertiary center, Ziekenhuis Oost Limburg (ZOL), 4 weeks after the second dose of the ChAdOx1-S vaccine.

Methods

Participants and sample handling

Health care workers who received the second dose of the SARS-CoV-2 vaccine ChAdOx1-S at the Hospital Oost Limburg (ZOL) hospital, a tertiary center in Belgium, were invited for blood sampling. The study was in accordance with the declaration of Helsinki, and approved by the local medical ethics committee and by the federal agency for medicines and health products (FAMHP). All participants gave written informed consent. Subjects with known coagulation defects or subjects below 18 years of age were excluded from the study.

Control samples were collected prior to the start of the COVID-19 pandemic after approval by the local medical ethics committee of the Maastricht University Medical Center and after written informed consent. Subjects with known coagulation defects, subjects below 18 years of age or individuals using anticoagulants were excluded from the study. As the vaccinated health care worker cohort consists primarily of women, the control group consisted of subjects that were matched for sex and age. Additionally, reference ranges for each parameter were determined in a group of 120 healthy individuals, consisting of 50% men, 37% women without oral contraceptives, and 13% women with oral contraceptives.

Blood was collected on 0.109 M sodium citrate (9 NC coagulation sodium citrate 3.2%, Greiner Bio-One GmbH, Austria) for analysis of hemostatic parameters. Serum (BD vacutainer SST II Advance, BD, UK) was prepared for the detection of anti-SARS-CoV-2 antibodies. Citrated blood was centrifugated twice at 2,821 g for 10 min to prepare platelet poor plasma. Serum tubes were centrifugated once at 2,821 g for 10 min to prepare serum. All samples were aliquoted and stored at −80°C until further use.

COVID-19 related tests

SARS-CoV-2 anti-S antibodies were measured using the quantitative Elecsys anti-RBD, in addition to semi-quantitative Elecsys anti-N (both measuring total immunoglobulin levels) on the cobas e801 analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Results for the quantitative Elecsys anti-RBD

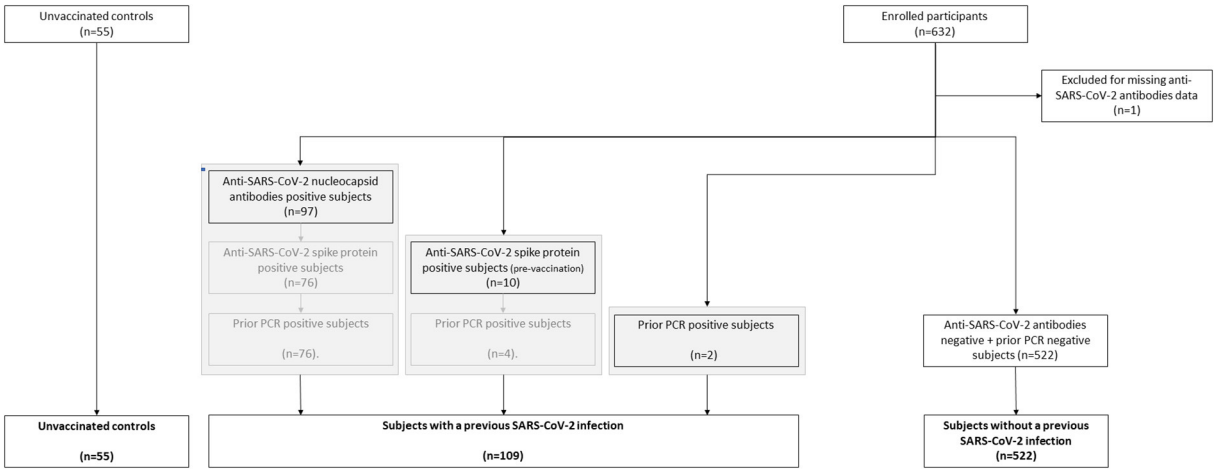

FIGURE 1
Flow chart of the classification of subjects vaccinated with ChAdOx1-S with and without prior COVID-19.

antibodies are reported as concentrations (U/mL), with a manufacturer's cut-off > 0.8 U/mL considered as positive. Results for the Elecsys anti-N antibodies are reported as a cut-off index (signal sample/cut-off or signal calibrator), with values > 1 considered as positive. A previous history of COVID-19 could be established by the presence of anti-SARS-CoV-2 nucleocapsid antibodies (post-vaccination), the presence of anti-SARS-CoV-2 spike protein antibodies (pre-vaccination), a positive PCR test (pre-vaccination) or any combination (Figure 1).

Hemostatic parameters and coagulation factors

D-dimer, fibrinogen, antithrombin and FVIII were measured on the STA-R (Diagnostics Stago, Asnières, France), using STA-Liatest D-di Plus, STA-Liquid Fib, STA-Stachrom AT III and STA-Immunodef FVIII reagents (Diagnostics Stago, Asnières, France), respectively according to the manufacturer's specifications. Von Willebrand Factor (VWF) and active VWF levels were measured with an in house ELISA, as described in detail elsewhere (11). Functional α_2M levels were measured with an in-house method as previously described (12).

Thrombin generation

Thrombin generation (TG) was measured in platelet poor plasma by the calibrated automated thrombinography (CAT) method, using PPP reagent, PPP reagent low, Calibrator and FluCa kits (Diagnostics Stago, Asnières, France) according to the manufacturer's specifications. TG was measured in the presence or absence of thrombomodulin (TM; 50% inhibition of the peak height in pooled normal plasma) to test the sensitivity of the activated protein C (APC) system. TG curves and parameters were generated by the dedicated CAT software.

Measurement of interleukins

IL-6 and IL-10 concentrations were determined by ELISA using the BMS213 kit (Invitrogen, Waltham, Massachusetts) and BMS215 kit (Invitrogen, Waltham, Massachusetts), respectively, in accordance with the manufacturer's instructions.

Clinical adverse effects

All health care workers in Hospital Oost-Limburg were invited to report adverse effects after SARS-CoV2 vaccination through a questionnaire at day 8 after each vaccine dose. Clinical symptoms were reported as none, mild, moderate or severe for each symptom, except insomnia, which was classified as none, mild or moderate, and anaphylaxis and facial paralysis, which was reported as present or not present. For further analysis, clinical symptoms were either classified as "injection site symptoms" (tenderness of the arm and redness of the arm), or "systemic symptoms" (fatigue, headache, muscle pain, chills, fever, joint pain, insomnia, nausea, swollen lymph nodes, facial paralysis, and anaphylaxis).

Statistical analyses

Differences between groups were determined using the Chi^2 test for categorical data and ANOVA or Kruskal-Wallis

TABLE 1 General characteristics of controls subjects and subjects vaccinated with ChAdOx1-S.

	Control subjects	Vaccinated subjects without previous SARS-CoV-2 infection	Vaccinated subjects with previous SARS-CoV-2 infection	*P*-value
N	55	522	109	
Age, years (SD)	39 (9)	40 (9)	39 (10)	n.s.
Sex, number of men (%)	4 (7%)	81 (15.5%)	11 (10.0%)	n.s.

Data are displayed as mean and standard deviation or as percentages in the case of categorical variables. Differences between the groups were analyzed by ANOVA. A p-value below 0.05 was considered statistically significant.

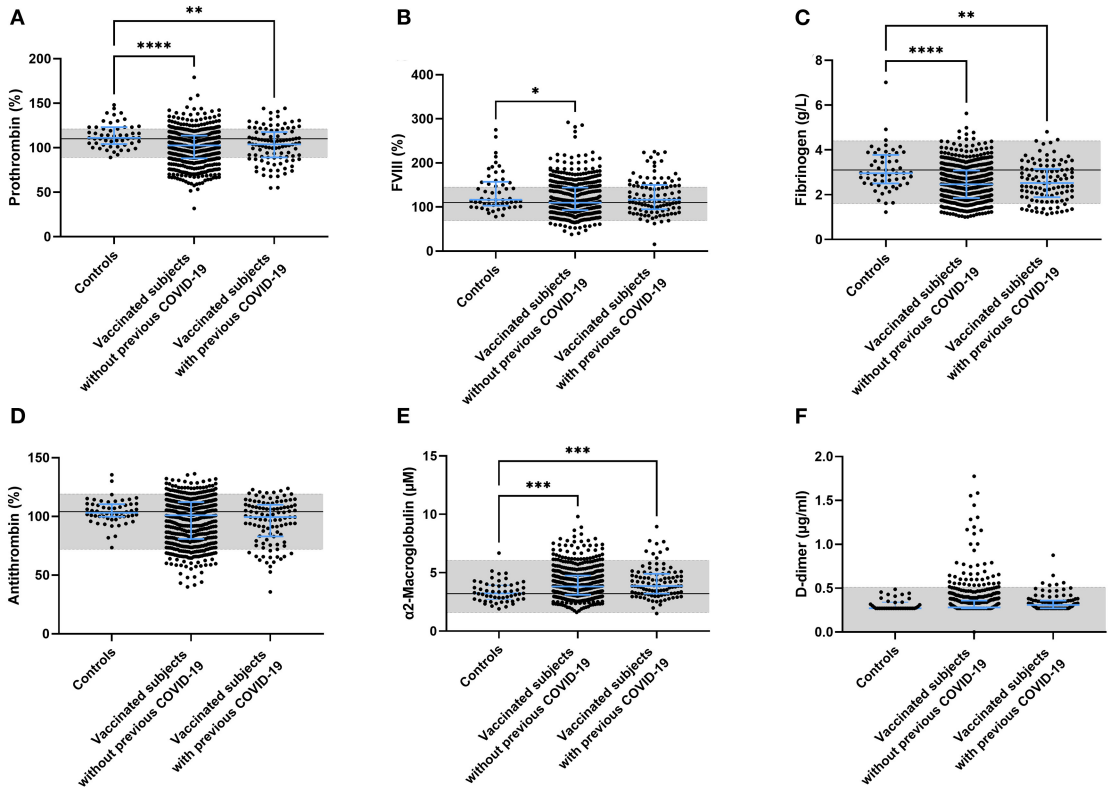

FIGURE 2
Coagulation factor levels in controls and subjects vaccinated with ChAdOx1-S with and without prior COVID-19 infection. Plasma levels of prothrombin **(A)**, FVIII **(B)**, fibrinogen **(C)**, antithrombin **(D)**, α2-macroglobulin **(E)** and D-dimer **(F)** were quantified. Reference values for each test are indicated as the gray area in the dot plot. Differences between the groups were analyzed by ANOVA or Kruskal-Wallis analysis, depending on the distribution of the data. *P*-values below 0.05, 0.01, 0.001, and 0.0001 were marked as *, **, ***, and ****, respectively.

analysis for continuous data, depending on the distribution of the data. When comparing two groups, either the *t*-test or the Mann Whitney test was used instead. Statistical significance is indicated as *, **, and ***, respectively for *p*-values below 0.05, 0.01, and 0.001. Statistical analyses were performed in GraphPad Prism (version 8; GraphPad Software, San Diego, California), and IBM Statistical Package for Social Sciences (SPSS; version 25; SPSS Incorporated, Chicago, Illinois).

Results

We assessed the hemostatic profile in 631 health care workers vaccinated with SARS-CoV-2 vaccine ChAdOx1-S and in 55 controls (Table 1). The majority of the vaccinated population was female (85.4%) and the median age was 41 years. Age and sex were comparable in the control group, which consisted of 51 women (93%) with an average age of 39 years. In the vaccinated subjects, blood sampling was performed

on average 32 days (range 21–40 days) after the second dose of ChAdOx1-S. All vaccinated participants developed anti-SARS-CoV-2 spike protein antibodies. Anti-SARS-CoV-2 nucleocapsid antibodies, related to a previous COVID-19 infection, were present in 13.9% of the vaccinated subjects. A total of 109 participants (17.3%) suffered from COVID-19 in the past (Figure 1). In this subset of individuals, in which COVID-19 infection was confirmed during the infection with a PCR test, the median time between the COVID-19 infection and the blood drawing is 34 weeks (IQR 26–60 weeks). Furthermore, clinical symptoms were recorded during the study, and no thrombotic or hemorrhagic complication was reported after vaccination.

ChAdOx1-S vaccination and procoagulant factor levels

Multiple coagulation factor levels and hemostatic variables were determined in controls, vaccinated subjects and vaccinated subjects with a prior COVID-19 infection (Figure 2). Prothrombin and fibrinogen levels were significantly reduced after vaccination (−7.5%, $p < 0.0001$ and −16.9%, $p < 0.0001$), suggesting ongoing usage of coagulation factors. Many post-vaccination samples showed both prothrombin (42.1%) and fibrinogen levels (16.1%) outside of the normal range, which was significantly less in the control group (26.4% with $p = 0.026$, and 7.3% with $p = 0.081$, respectively; Figures 2A,C). FVIII levels were comparable between controls and vaccinated subjects (Figure 2B). Additionally, we found that 9% of the vaccinated subjects had D-dimer levels outside of the normal range, compared to none of the control subjects ($p = 0.047$).

ChAdOx1-S vaccination and anticoagulant factor levels

Plasma antithrombin levels did not differ between controls, vaccinated subjects and vaccinated subjects with a prior COVID-19 infection. As for prothrombin and fibrinogen, the levels of antithrombin were outside of the normal range in 23.9% of

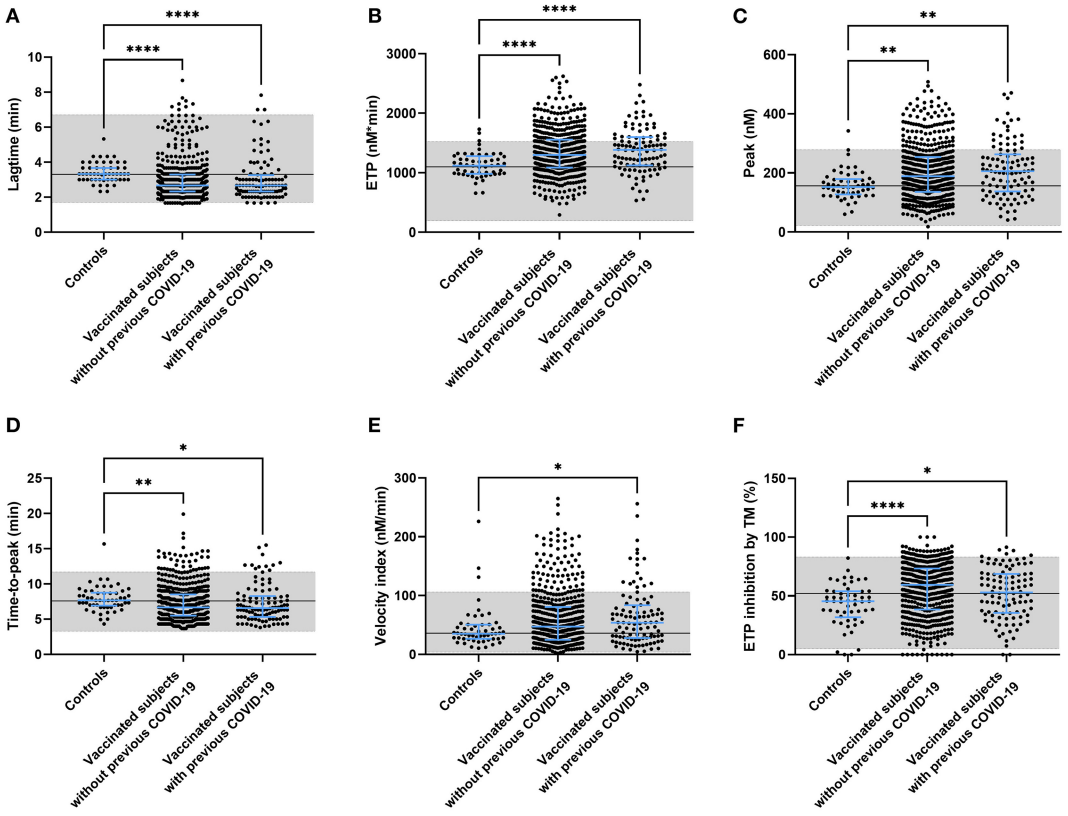

FIGURE 3
Thrombin generation parameters in controls and subjects vaccinated with ChAdOx1-S with and without prior COVID-19 infection. Thrombin generation was measured at 5 pM tissue factor and the lag time **(A)**, ETP **(B)**, peak height **(C)**, time-to-peak **(D)**, velocity index **(E)**, and the inhibition of the ETP by the addition of thrombomodulin **(F)** were quantified. Reference values for each parameter are indicated as the gray area in the dot plot. Differences between the groups were analyzed by ANOVA or Kruskal-Wallis analysis, depending on the distribution of the data. *P*-values below 0.05, 0.01, 0.001, and 0.0001 were marked as *, **, ***, and ****, respectively.

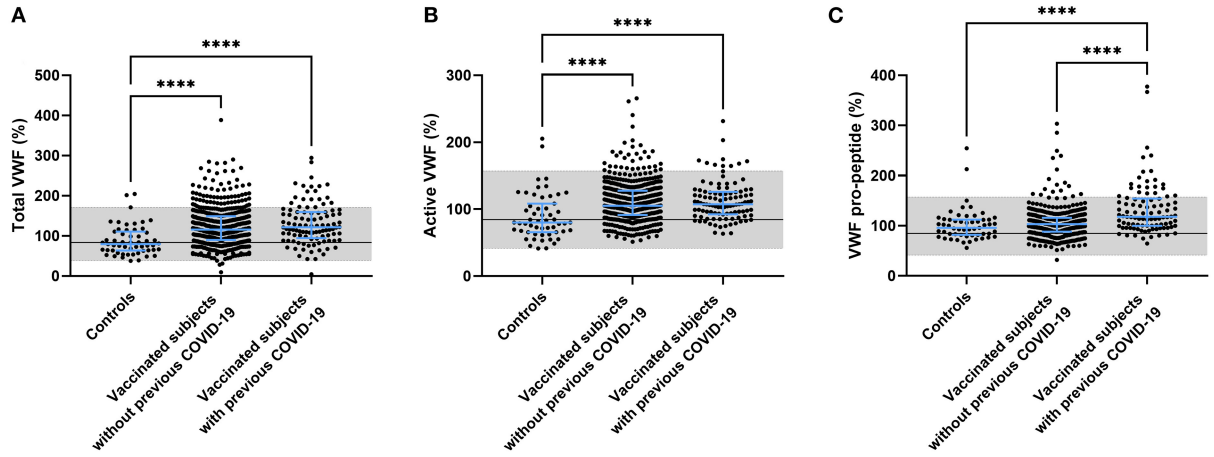

FIGURE 4
Endothelial activation in controls and subjects vaccinated with ChAdOx1-S with and without prior COVID-19 infection. Endothelial activation was assessed by the measurement of total VWF **(A)**, active VWF **(B)** and VWF pro-peptide **(C)**. Reference values for each test are indicated as the gray area in the dot plot. Differences between the groups were analyzed by ANOVA or Kruskal-Wallis analysis, depending on the distribution of the data. *P*-values below 0.05, 0.01, 0.001, and 0.0001 were marked respectively.

the vaccinated individuals (compared to 3.6% in the control group ($p = 0.001$; Figure 2D). In contrast to the reduced fibrinogen levels, the plasma levels of another acute phase protein and thrombin inhibitor, α_2M, increased significantly after vaccination in both the group without (+ 20.0%, $p = 0.0008$; Figure 2F) and the group with prior COVID-19 infection (+ 23.1%, $p = 0.0006$; Figure 2E).

ChAdOx1-S vaccination and thrombin generation

To better understand the effect of these changes in coagulation factor levels, we used the thrombin generation assay as an indicator of global coagulation. Figure 3 displays the thrombin generation parameters obtained after stimulation with 5 pM tissue factor (TF). Stimulation with 1 pM TF gave similar results. Both time-dependent parameters lag time and time-to-peak were significantly shorter in vaccinated subjects than controls (−12.7%, $p < 0.0001$ and −7.1% and $p < 0.0001$), as well as in vaccinated subjects with prior COVID-19 compared to control (−11.5%, $p < 0.0001$ and −7.3% and $p = 0.0003$) (Figures 3A,D). The endogenous thrombin potential, which is a marker of the overall clotting ability of a subject, was increased in vaccinated subjects without (+ 18.7%, $p < 0.0001$; Figure 3B) and with a history of COVID-19 (+ 22.4%, $p < 0.0001$). A similar effect was seen for the thrombin generation peak height, which was increased by 27.1% ($p = 0.0034$) and 32.6% ($p = 0.0015$) in vaccinated subjects with and without prior COVID-19 infection, respectively (Figures 3C,E). Additionally, we investigated the effect of the natural anticoagulant activated protein C system *via* the addition of thrombomodulin and the subsequent quantification of its inhibitory action on the endogenous thrombin potential (ETP) (Figure 3F). Vaccinated subjects had a significantly larger response to the addition of thrombomodulin, regardless of their history of COVID-19 infection.

ChAdOx1-S vaccination and von willebrand factor

Another component that is known to play a role in the pathogenesis of thrombosis is the vessel wall. An important biomarker of endothelial activation is von Willebrand factor (VWF). We quantified the amount of circulating total VWF, active VWF (VWF in a GPIb-binding conformation) and VWF pro-peptide in controls and vaccinated subjects. Both total and active VWF were significantly higher in vaccinated subjects (+ 39.5%, $p < 0.0001$ for total VWF and +24.1 %, $p < 0.0001$ for active VWF) and this effect seemed to be more pronounced in vaccinated subjects with a prior COVID-19 infection (+ 47.8%, $p < 0.0001$ for total VWF and +26.2 %, $p < 0.0001$ for active VWF; Figures 4A,B) although it did not reach significance. We did find that VWF pro-peptide was significantly increased in subjects with a history of COVID-19 compared to controls (+ 30.7%, $p < 0.0001$) and compared to vaccinated subjects without prior COVID-19 (+27.8%, $p < 0.0001$) (Figure 4C).

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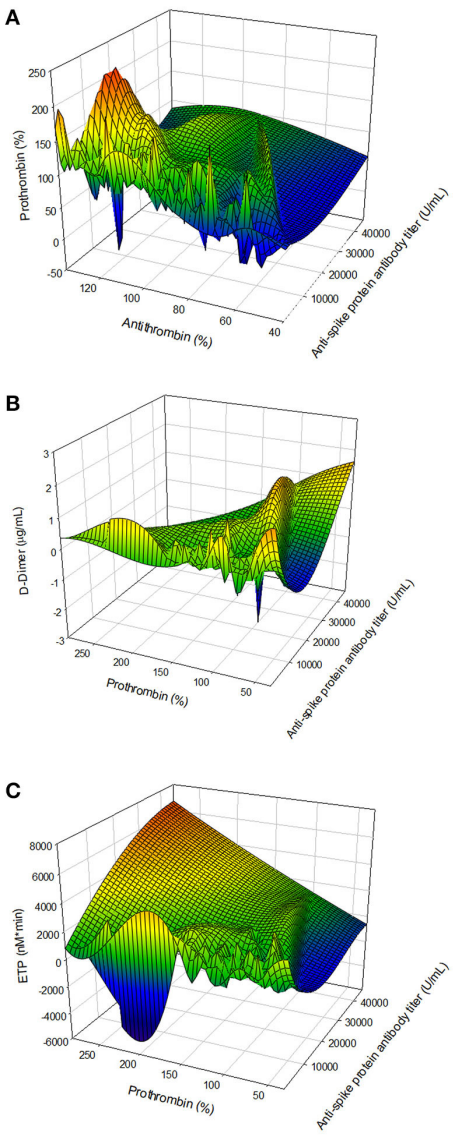

FIGURE 5
The association of the anti-SARS-CoV-2 spike protein antibody titer and hemostatic parameters. **(A)** The relationship antibody titer and coagulation factor levels: antithrombin and prothrombin. **(B)** The association of antibody titer and coagulation factor consumption: prothrombin and D-dimer. **(C)** The relation of antibody titer and the thrombin generation potential, quantified by the ETP and prothrombin level.

The association of anti-SARS-CoV-2 spike protein antibody titer and hemostatic parameters

We observed that vaccination with ChAdOx1-S affected several parts of the hemostatic system. To further investigate this, we studied a possible relationship with the titer of the anti-SARS-CoV-2 spike protein antibody titer with hemostatic parameters. Figures 5A–C shows that a higher anti-SARS-CoV-2 spike protein antibody titer is associated with a lower prothrombin and antithrombin level. Additionally, in subjects with a high anti-spike protein titer, D-dimer levels were higher when prothrombin levels were low. Assessing thrombin generation, the endogenous thrombin potential is higher in subjects with a high anti-spike protein titer compared to subjects with a low titer, at comparable prothrombin levels.

ChAdOx1-S vaccination and interleukin levels

Furthermore, in the pathogenesis of COVID-19 infection, IL-6 and IL-10 have been implicated as a link between the immune system and coagulation. We investigated the role of the immune system in the apparent haemostatic changes after vaccination with ChAdOx1-S by measuring interleukin (IL) 6 and IL-10 in a subset of the population. IL-6 was comparable between the vaccinated population and the control group (data not shown). On the contrary, IL-10 was significantly higher in the group of vaccinated subjects, compared to control subjects (9.29 pg/mL ± 16.9 pg/mL vs. 2.43 pg/mL ± 3.58 pg/mL, $p = 0.032$; data not shown).

The association of post-ChAdOx1-S vaccination clinical symptoms and hemostatic parameters

Clinical symptoms after the second round of vaccination were recorded. We investigated whether there was an association between clinical adverse effects related to the vaccination with coagulation factor levels (Table 2) and thrombin generation parameters (Figure 6). Prothrombin levels were significantly lower (100% ± 19% vs. 104% ± 19%, $p = 0.027$) in subjects that reported injection site symptoms compared to subjects that did not report injection site symptoms. Antithrombin levels were lower as well in subjects that reported injection site symptoms (95% ± 18% vs. 101% ± 19%, $p = 0.005$). Fibrinogen, α_2Macroglobulin, FVIII, and D-dimer levels were comparable between vaccinated subjects with and without injection site symptoms. Moreover, whereas total VWF and active VWF levels were the same in subjects with and without injection site symptoms, VWF pro-peptide levels were 13% lower in subjects with injection site symptoms ($p < 0.001$). Subjects that reported systemic symptoms had significantly lower FVIII levels compared to subjects that did not report systemic symptoms (117% ± 33% vs. 127% ± 41%, $p = 0.027$) Interestingly, increasing severity of injection site symptoms (Figures 6A,C,E,G,I), but not systemic symptoms (Figures 6B,D,F,H,J), is associated with an increasingly more

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TABLE 2 The association of coagulation factor levels and clinical symptoms after the second round of vaccination as reported by subjects vaccinated with ChAdOx1-S with and without prior COVID-19 infection.

Coagulation factor	Injection site symptoms			Systemic symptoms		
	No Symptoms	*Symptoms*	*P-value*	*No Symptoms*	*Symptoms*	*P-value*
Prothrombin, % (SD)	104 (19)	100 (19)	*0.027*	103 (19)	100 (19)	*n.s.*
Antithrombin, % (SD)	101 (19)	95 (18)	*0.005*	98 (20)	97 (19)	*n.s.*
Fibrinogen, g/L (SD)	2.60 (0.81)	2.51 (0.84)	*n.s.*	2.59 (0.86)	2.54 (0.84)	*n.s.*
α2-Macroglobulin, μM (SD)	3.97 (1.26)	4.15 (1.27)	*n.s.*	3.98 (1.18)	4.14 (1.30)	*n.s.*
FVIII, % (SD)	126 (42)	120 (38)	*n.s.*	127 (41)	117 (33)	*0.027*
D-Dimer, μg/mL (SD)	0.30 (0.04)	0.30 (0.04)	*n.s.*	0.30 (0.04)	0.30 (0.04)	*n.s.*
VWF, % (SD)	125 (52)	125 (45)	*n.s.*	128 (45)	120 (41)	*n.s.*
active VWF, % (SD)	112 (26)	112 (27)	*n.s.*	114 (26)	111 (27)	*n.s.*
VWF pro-peptide, % (SD)	131 (45)	114 (33)	*<0.001*	125 (43)	117 (35)	*n.s.*

Data are displayed as mean and standard deviation or as percentages in the case of categorical variables. Differences between the groups were analyzed by the student T-test or the Mann Whitney test, depending on the distribution of the data. A p-value below 0.05 was considered statistically significant.

procoagulant thrombin generation profile (Figure 6). This is reflected by a higher peak height (+ 8.3, + 19.7, and +35.2% in subjects with mild, moderate and severe injection site symptoms compared to symptom free subjects (p = 0.038) (Figure 6C). Moreover, the time-to-peak shortened with increasing symptoms severity (p = 0.028) (Figure 6E), and the velocity index increased from 47 nM/min ± 32 nM/min in symptoms free subjects, to 59 nM/min ± 41 nM/min in subjects with mild symptoms, 73 nM/min ± 55 nM/min in subjects with moderate symptoms and 89 nM/min ± 56 nM/min in subjects with severe symptoms (p = 0.0003) (Figure 6I).

Discussion

Vaccination against COVID-19 has been adopted all over the world with a few exceptions. It has been shown that in general vaccination with either one of the four vaccines approved by the FDA/EMA - ChAdOx1-S (Vaxzevria; Astra Zeneca), Ad26.COV2-S (Janssen COVID-19 Vaccine; Johnson and Johnson) and two COVID-19 mRNA Vaccines (Comirnaty and Spikevax, respectively from Pfizer and Moderna), is safe (13–16). Despite the acceptable safety records, rare incidences of thrombosis after vaccination of which VITT in relation to COVID-19 vaccination have reached the news worldwide. Interestingly, and less well-known, a large Scandinavian study revealed that the risk of venous thromboembolic events is almost double in subjects after receiving the ChAdOx1-S vaccine, as 59 events occurred in their cohort (N = 281,264), while 30 events were expected (17).

In our study we investigated possible changes in haemostasis after full vaccination with ChAdOx1-S related to an increased risk of thrombosis. We studied 631 individuals after receiving ChAdOx1-S and found three clusters of changes: (1) Lower coagulations levels indicating usage of coagulation factors; (2) Increased and faster thrombin generation indicating a more active coagulation system; and (3) increased VWF levels indicating activation of the vascular wall. All three clusters together show that the haemostatic system is shifted to a more procoagulant state compared to unvaccinated controls.

Several mechanisms may explain these changes at 4 weeks after vaccination. First, vaccination triggers antibody formation and our results indicate the thrombin generation profile becomes more procoagulant in subjects vaccinated with ChAdOx1-S, shown by the increased endogenous thrombin potential both in vaccinated subjects with or without prior COVID-19 infection. Other groups have shown that ChAdOx1-S vaccination causes a transient increase in TG (18), although they could not detect a significant difference between vaccination subjects and unvaccinated controls (19). Moreover, our results show that the endogenous thrombin potential is correlated with the magnitude of anti-spike protein antibody titer. The more procoagulant TG profile is further reflected in an increased TG peak height in vaccinated subjects and shortened time-dependent TG parameters, which indicates that the TG potential is not only increased, but that TG is also triggered faster.

We did not find a direct cause of the increase of the thrombin generation profile, although significant associations exist between the TG parameters and the titer of the anti-spike protein antibodies. Coagulation factors with established procoagulant effects such as prothrombin, FVIII and fibrinogen are not increased or even decreased upon vaccination with ChAdOx1-S (12, 20–22). Moreover, vaccinated subjects are more sensitive to the anticoagulant effect of thrombomodulin (TM) than controls, and therefore, the changes found in TG cannot be attributed to reduced activation of the activated

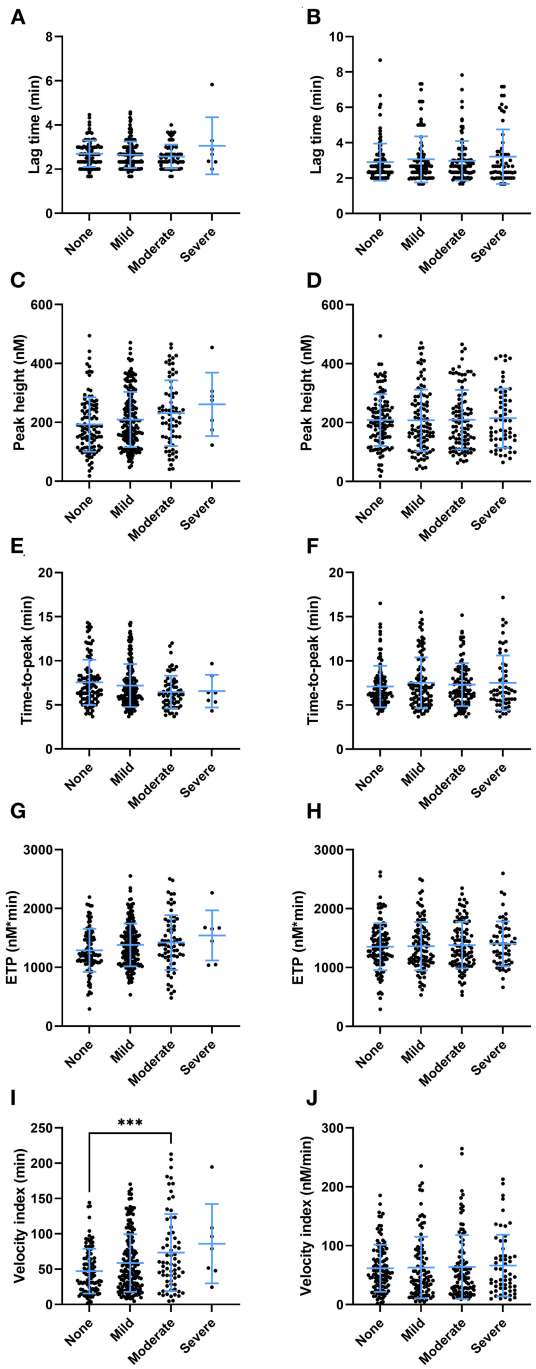

FIGURE 6
The association of thrombin generation parameters and clinical symptoms after the second round of vaccination as reported by subjects vaccinated with ChAdOx1-S with and without prior COVID-19 infection. **(A,B)** The lag time did not differ significantly at the increasing levels of injection site symptoms **(A)** or systemic symptoms **(B)**. The peak height differed significantly between subjects with increasing injection site symptoms ($p < 0.038$), although the study was underpowered to detect differences between specific groups in *post hoc* analysis **(C)**. Increasing systemic symptoms did not result in differences in peak height **(D)**. **(E,F)** The time-to-peak differed significantly *(Continued)*

FIGURE 6
between the categories of injection site symptoms ($p = 0.029$) **(E)**, but not systemic symptoms **(F)**. **(G,H)** The ETP was not significantly affected by the various levels of injection site symptoms **(G)** or systemic symptoms **(H)**. **(I)** The velocity index was significantly increased in subjects with more injection site symptoms ($p < 0.001$) **(I)**, but not systemic symptoms **(J)**. Data are displayed as mean and standard deviation or as percentages in the case of categorical variables. Differences between the groups were analyzed by ANOVA or Kruskal-Wallis analysis, depending on the distribution of the data. A *p*-value below 0.05 was considered statistically significant and *** indicates a *p*-value below 0.001.

protein C pathway. Nevertheless, the reduction of procoagulant factor levels in combination with an increased D-dimer and an increased thrombin generation potential, point toward a mild consumption of coagulation factors *in vivo* due to ongoing activation of the coagulation system.

Second, our results show that vaccination induces endothelial activation, as reflected by an increase of total VWF and active VWF in the blood stream, and the elevated levels of VWF pro-peptide indicate that this is a recent effect in the post-vaccination period. In severe SARS-CoV-2 infections, the vascular endothelium is the cornerstone of organ dysfunction and recent data have emphasized the crucial role of endothelial cells in vascular dysfunction, immune-thrombosis and inflammation (23, 24). Moreover, vascular activation is a strong predictor of mortality in COVID-19 disease on the ICU (25). Post COVID-19 infection endothelial activation is still measurable at 3 months after hospital discharge (17, 26) and may comprise part of the long COVID syndrome (27). It remains unknown which mechanisms trigger late endothelial activation following vaccination, mimicking elements of COVID-19.

Third, others have shown that single cell sequencing of peripheral blood mononuclear cells before and at 28 days after vaccination with an inactivated SARS-CoV-2 vaccine showed consistent changes in gene expression levels in many different immune cell types, also including inflammatory pathways regulated by the NFkB system. These changes, reflecting the cellular immune response to vaccination, were associated with, amongst others, detectable alterations in inflammatory mediators and global coagulation tests, suggesting that immune cell priming could also have protracted effects on hemostasis, maybe unrelated to antibody responses (28). One of the main procoagulant drivers, tissue factor, is regulated through the NFkB system, which is amplified in the study by Liu and colleagues, suggesting that monocytic, or extracellular vesicle mediated expression of tissue factor, may be an additional trigger of thrombin generation observed in our study. The fact that the key cytokine Il-6, that shows an early small peak 1 day after vaccination with ChAdOx1-S (26), is no longer elevated at 4 weeks, makes it less likely that the procoagulant changes in this study are the direct consequence of pro-inflammatory

cytokine release. However, alike the IL-10 increase in patients with a COVID-19 infection, IL-10 was found to be elevated as well in vaccinated subjects, regardless of the history of COVID-19 infection in the past. IL-10 is known to attenuate coagulation and fibrinolysis (29). Strangely enough, the role of IL-10 is not fully known and somewhat ambiguous in COVID-19. As we found increased IL-10 levels, this further indicates that the immune system is still active, which might also contribute to the prolonged hemostatic changes that we have found in the vaccinated population.

A limitation of this study is that it mainly studies the effect of ChAdOx1-S vaccination in women, as the predominant part of the healthy workers are female. Subsequently, an unvaccinated pre-COVID-19 control group was chose for result comparison. These samples were stored longer at −80°C than the samples of vaccinated subjects, because it was difficult at the time of the study to include controls that were unvaccinated and naïve to the SARS-CoV-2 virus. Sex is known to affect thrombin generation and coagulation factor levels and function. Therefore, we included reference values obtained in a more heterogenous group of 120 healthy adults (50% men, 37% women without oral contraceptives and 13% women with oral contraceptives) for each parameter measured in this study. Moreover, due to the large group size, some results are statistically significant, although the clinical significance of small changes in these parameters might be debatable. Additionally, there was were not much data available on the background of the vaccinated subjects and unvaccinated controls, such as body mass index, smoking status, or oral contraceptive use.

The net impression of our findings is that 4 weeks after vaccination with ChAdOx1-S, a substantial subset of individuals still show signs of endothelial and hemostatic activation that may explain at least part of the observed apparent increase in thromboembolic events after vaccination. Whether these effects are specific for this vaccine and would also appear to some extent after vaccination with Ad26.COV2-S or even the mRNA vaccines, remains to be determined. VITT is a very rare event occurring earlier on after vaccination and although hemostatic changes like the ones we observed could theoretically contribute to a subject's sensitivity to develop immune thrombosis, this conclusion cannot be drawn from the present data. Other rare thromboembolic events, apparently unrelated to VITT (eg without thrombocytopenia) have also been noted after vaccination (17, 30) and presumably, the individual variation in immune and procoagulant responses, could contribute to the risk of thrombosis.

It is obvious from the cumulative literature data that the risks of COVID-19 by far exceed the very small risks of (immune) thrombosis, whether VITT or not, and our data should in no way be used to rally against the benefits of vaccination. Nevertheless, the mechanistic data from this study underline how closely immunity and hemostatic systems are intertwined, illustrating the evolutionary importance of both systems in defense against invading pathogens.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Comite Medische Ethiek Ziekenhuis Oost-Limburg. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BL, LH, RH, HC, and DS supervised the study. MN, MR, DH, and HS performed experiments and analyzed the data. DM, ST, KV, JP, PV, RD, ML, and TF collected patient samples, processed the data and critically read the manuscript. RdL-K analyzed the data and prepared the figures. BL and RdL-K drafted the manuscript. HS, MN, RH, LH, HC, and DS critically revised it. All authors contributed to the article and approved the submitted version.

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Conflict of interest

BL, RdL-K, MR, DH, and MN are employees of Synapse Research Institute, part of Diagnostica Stago. HC received funding for research from Bayer and Pfizer; compensation fees for consultancy and advisory boards from Daaichi, Pfizer, Leo, Bayer, Galapagos, Anthos, Alexion, and Alveron; shareholder from Coagulation profile; all benefits were transferred to the CARIM institute to support investigator-initiated research.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Assessing the individual roles of FII, FV, and FX activity in the thrombin generation process

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Thrombin generation (TG) is known as a physiological approach to assess the hemostatic function. Although it correlates well with thrombosis and bleeding, in the current setup it is not sensitive to the effects of fluctuations in single coagulation factors. We optimized the calibrated automated thrombinography (CAT) method to quantify FII, FV and FX activity within the coagulation system. The CAT assay was fine-tuned for the assessment of FII, FV and FX by diluting the samples in FII-, FV-, or FX-deficient plasma, respectively, and measuring TG. Plasma FII levels correlated linearly with the ETP up to a plasma concentration of 100% FII. FV and FX levels correlated linearly with the peak height up to a plasma level of 2.5% FV and 10% FX, respectively. Sensitized CAT protocols were designed by adding a fixed volume of a pre-diluted patient sample to FII, FV, and FX deficient plasma in TG experiments. This approach makes the TG measurement dependent on the activity of the respective coagulation factor. The ETP or peak height were quantified as readouts for the coagulation factor activity. The intra- and inter-assay variation coefficients varied from 5.0 to 8.6%, and from 3.5 to 5.9%, respectively. Reference values were determined in 120 healthy subjects and the assays were clinically validated in 60 patients undergoing coronary artery bypass grafting (CABG). The sensitized CAT assays revealed that the contribution of FII, FV, and FX to the TG process was reduced after CABG surgery, leading to reduced prothrombin conversion and subsequently, lower TG.

KEYWORDS

thrombin generation, factor V, prothrombin, factor X, coagulation factors, calibrated automated thrombinography

Introduction

The Calibrated Automated Thrombinography (CAT) is a global hemostasis assay that measures the thrombogenic potential of an individual (1). Since the introduction of the semi-automatic CAT method approximately 30 years ago (2), thrombin generation (TG) has gained popularity in assessment of coagulation (3–8). Over the last decade, many studies have shown the association of low TG and bleeding (9, 10), and high

TG and thrombosis (11, 12). As a result of the clinical usefulness of TG in the prediction of bleeding and thrombosis risk (6), and the monitoring of patients receiving anticoagulation (13, 14) or transfusion products (15, 16), fully automated systems have been developed to measure TG in clinical laboratory settings (17, 18).

The TG test is a global coagulation assay, and therefore it is more sensitive to fluctuations in certain coagulation factors than others. Prothrombin and antithrombin, for example, are known to have a large effect on TG, whereas FV and FVIII only cause pronounced changes in the TG profile if their levels are far below the normal range (19). Moreover, the original CAT test results give the combined result of the interplay of all plasmatic components of the coagulation system, and subsequently changes in regular TG usually cannot be attributed to a specific coagulation factor (20, 21). Nevertheless, the function of individual coagulation factors is important, e.g., for monitoring treatment. Moreover, there is an increasing interest in the relationship between specific coagulation factor activity and thrombotic/bleeding diseases (22–25).

With modification, it has been shown that the CAT has the potential to measure the recombinant FVIII and FIX potency in hemophilia plasma (26–29). These findings demonstrate that there is potential to sensitize the CAT assay to individual coagulation factors. In this study, we set out to investigate the possibility to sensitize the CAT assay to the components of the prothrombinase complex (FII, FV, and FX).

Materials and methods

Patient and healthy subject samples

Healthy subjects ($n = 120$) were enrolled in the study after giving their full informed consent according to the Declaration of Helsinki and after approval of the local medical ethics board of Maastricht University Medical Center. Blood was collected on 3.2% sodium citrate (BD Vacutainer System). Platelet poor plasma was obtained by centrifugation twice at 2,630 g for 10 min and stored at −80°C until further use.

Additionally, 60 patients undergoing coronary artery bypass grafting (CABG) surgery were enrolled in the study. Blood was collected at two time points: before the start of the surgery and after the surgery and protamine administration. Blood was collected on 3.2% sodium citrate (BD Vacutainer System). Platelet poor plasma was obtained by centrifugation twice at 2,630 g for 10 min and stored at −80°C until further use.

Thrombin generation

TG without adaptations, as shown in Figure 1, was measured using the CAT method in a 96-well plate (Thrombinoscope B. V., Maastricht, Netherlands) (30). Each well contained 20 μL PPP Reagent High or 20 μL calibrator, 80 uL of platelet poor plasma sample, and 20 μL FluCa. Calibrator, PPP Reagent High and FluCa kits were purchased from Diagnostica Stago (France). Data were processed with dedicated software (Thrombinoscope B. V, Maastricht, Netherlands).

Coagulation factor sensitized CAT assay

Coagulation factor sensitized CAT assays were performed as TG experiments, using a mix of prediluted plasma samples and commercial deficient plasmas. Pre-diluted plasma samples were prepared by diluting healthy individual or patient plasma samples in commercial plasma deficient in specific coagulation factors. The optimal conditions for plasma pre-dilution during the TG experiment and the ideal TG read-out parameter for the individual assays were determined separately for each assay, which resulted in three different measurement protocols for the coagulation factor sensitized CAT assays (Supplementary Figure 1). FII, FV, and FX deficient plasmas were purchased from Haematologic Technologies, Vermont, USA.

FII sensitized CAT assay

Each well of the 96-well plate contained 10 μL of undiluted patient sample, 70 μL of FII deficient plasma, 20 μL trigger mix and 20 μL FluCa. Calibration wells contained 10 μL of calibration plasma samples, which were prepared by mixing standard plasma to achieve calibration samples concentrations of 20, 40, 60, 80, 100, and 200% FII. TG was measured as described above, and a calibration curve was prepared based on the plasma FII activity and the corresponding ETP, allowing the calculation of the FII activity of patient plasma samples.

FV sensitized CAT assay

In the FV sensitized CAT assay, patient samples were diluted 1:20 in buffer. Each well contained 10 μL of diluted plasma sample, 80 μL of FV deficient plasma, 10 μL trigger mix and 20 μL FluCa. Calibration samples were prepared by mixing standard plasma with FV deficient plasma to achieve sample concentrations of 0.625, 1.25, 2.5, 5, and 10%. For patient samples, plasma was prediluted 1:20 to ensure that the FV levels within the calibrated range. TG was measured as described above, and a calibration curve was prepared based on the plasma FV activity and the corresponding peak height, allowing the calculation of the FV activity of patient plasma samples.

FX sensitized CAT assay

Each well of the 96-well plate contained 5 μL of patient sample, 75 μL of FX deficient plasma, 20 μL trigger mix

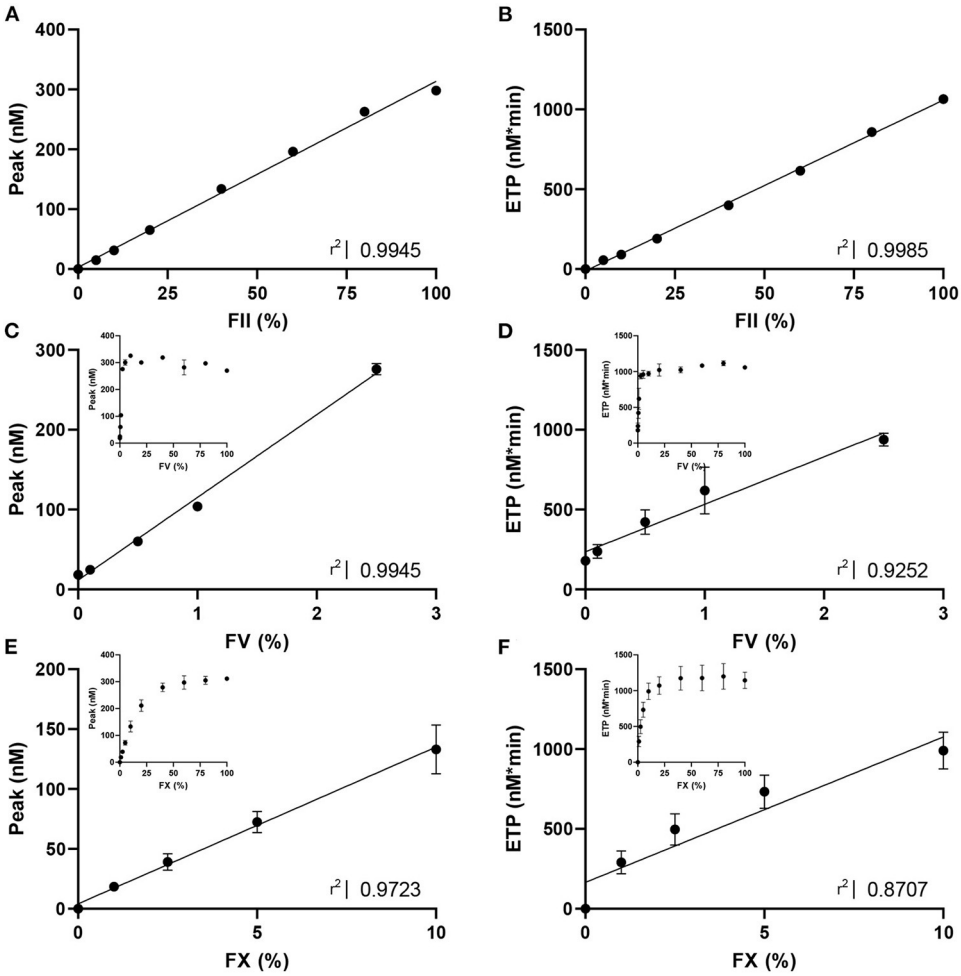

FIGURE 1
The dose response effect of FII, FV and FX on thrombin generation. TG triggered with PPP reagent high was measured in **(A,B)** FII-deficient plasma with varying concentrations of standard plasma (0, 5, 10, 20, 40, 60, 80, and 100% FII); **(C,D)** FV-deficient plasma with varying concentrations of standard plasma (0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 40, 60, 80, and 100% FV); and **(E,F)** FX-deficient plasma with varying concentrations of standard plasma (0, 1, 2.5, 5, 10, 20, 40, 60, 80, and 100% FX). Data are shown as the mean ± standard deviation and linear regression R^2 values are shown. For FV and FX, the linear part of the dose-response curve is analyzed with linear regression, and the whole range of ETP and peak are shown as insert in each panel. Statistical significance of the correlations was determined by Spearman correlation analysis, and all *p*-values were lower than 0.05.

and 20 μL FluCa. Calibration wells contained 5 μL of calibration plasma samples instead of patients samples, which were prepared by mixing standard plasma to achieve calibration samples concentrations containing 0, 25, 50, 100, and 150% FX. TG was measured as described above, and a calibration curve was prepared based on the plasma FX activity and the corresponding peak height, allowing the calculation of the FX activity of patient plasma samples.

Statistical analysis

Statistical analysis was performed in Graphpad Prism 5.0 (GraphPad Software, Inc., California). Linear curve fit was used to establish the calibration curves. The sensitivity was defined as the lowest coagulation factor activity level generating a signal that could be statistically differentiated from the baseline activity in factor-deficient plasma. A *p*-value below 0.05 was considered statistically significant. Reference values were determined as the range between 2.5th and 97.5th percentile values determined in 120 healthy subjects. Differences between pre- and post-CABG samples were assessed using the Wilcoxon matched-pairs signed rank test.

Results

To design CAT assays sensitized to the activity of FII, FV and FX, we first studied the dose-response effect of these

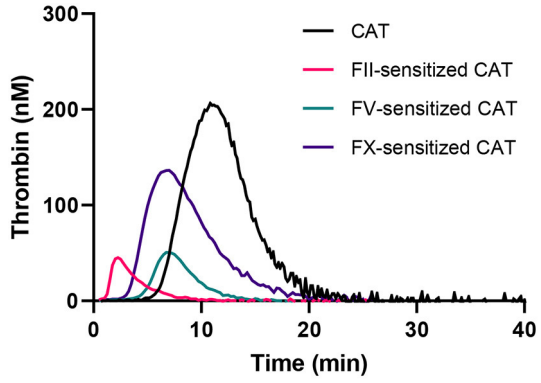

FIGURE 2
Thrombin generation curves generated in the standard CAT assay and the FII, FV, and FX sensitized CAT assays.

coagulation factors on 'regular' TG (Figure 1). TG was measured using PPP Reagent High in FII, FV or FX-deficient plasma spiked with increasing amounts of standard plasma, to achieve FII, FV or FX levels ranging from 0 to 100%, respectively. FII increased the ETP and peak dose-dependently for the whole range of concentrations (Figures 1A,B) and did not affect the lag time significantly. Complete deficiency in FII rendered an undetectable TG trace. In contrast, FV increased the peak height and ETP dose-dependently in the lower range (0–2.5% FV), but reached a plateau, respectively at 10% FV for the peak height and 20% FV for the ETP (Figures 1C,D). Additionally, the lag time decreased as FV increased. FX showed a similar effect on TG as FV, with a positive dose-dependent effect on ETP and peak height (Figures 1E,F). Alike FV, FX dose-dependently shortened the lag time.

The obtained data was used to design CAT assays sensitized to either FII, FV or FX, by mixing (prediluted) patient plasma with FII, FV or FX deficient plasma, respectively. This approach enables us to make the TG measurement dependent on the patient-sample derived FII, FV or FX, and allows us to study the contribution of these specific factors to the thrombin generation measurement. Three separate protocols, as described in the methods section, were designed to ensure the most optimal determination of the contribution of FII, FV, and FX to TG. In the "regular" TG setup, the FII level showed the best linear correlation with the ETP, and FV and FX showed the best linear correlation with peak height in the low concentration range. Therefore, the ETP was used as the readout for the FII sensitized CAT assay, whereas the peak height was used for analysis of the FV and FX sensitized CAT assays. The correlation between FV and the peak height is only linear up to 2.5% FV. Subsequently, the patient plasma samples need to be prediluted for the FV-sensitized CAT assay, to achieve a final FV level below 2.5% during the TG measurement. Similarly, the plasma FX levels is linearly correlated with the peak height up to a level of 10%, and in the FX-sensitized CAT assay, the plasma is prediluted accordingly. The correlation of the ETP and FII was linear between 0 and 200% FII. Subsequently, patient samples do not need to be pre-diluted for the sensitized CAT assays. Figure 2 illustrates the appearance of the TG curves generated by the "regular" CAT method and the FII-, FV- and FX-sensitized CAT assays. The experimental variability of the coagulation factor sensitized CAT assays was determined by performing the assays in duplicate on 10 different days in a healthy donor sample (normal coagulation factor levels), and a sample from a patient using Vitamin K antagonists (low coagulation factor levels). A sample with low FV was prepared by diluting pooled normal plasma in FV deficient plasma, as Vitamin K antagonist therapy does not affect FV levels. For the FII sensitized CAT assay, the intra-assay coefficient of variation (CV_{intra}) was 5.3 and 5.0%, respectively for the low and normal plasma sample. The inter-assay coefficient of variation (CV_{inter}) were 4.8 and 5.9% for the low and normal sample, respectively. The CV_{intra} of the FV sensitized CAT assay were 8.7 and 8.6%, respectively for a low and normal plasma sample, and the CV_{inter} were 10 and 5.7%. The CV_{intra} of the FX sensitized CAT assay were 13.4 and 5.8%, in the low and the normal sample, respectively and the CV_{inter} were 16.0 and 3.5%. The lower limit of detection of the assays was 20, 0.25, and 10% respectively for the FII, FV and FX sensitized CAT assays.

Assay validation in healthy subjects

The FII, FV and FX sensitized CAT assays were performed in 120 healthy subjects (Figure 3). In healthy subjects, the inter-individual CV was 13.5, 24.9, and 18.1% for the FII, FV and FX sensitized CAT assay, respectively. Reference ranges were determined for the three assays as the 2.5th−97.5th percentile values in 120 healthy individuals (Figure 3). FII has the narrowest range in healthy subjects, ranging from 85 to 157%. FV values showed the widest range, from 61 to 152% and FX ranged from 79 to 159%. Additionally, we investigated the correlation of the specific FII, FV, and FX activities in the CAT assay to the parameters of the original CAT assay in these 120 healthy subjects (Figure 4A). Both FII and FX activity are significantly and positively associated with the TG peak height and ETP in undiluted healthy subject samples. Furthermore, FX activity is positively associated with the velocity index. FV activity did not show a clear association with the original TG parameters in healthy subjects. Moreover, we determined the correlation of the TG parameters generated by the standard CAT assay and the FII-, FV-, and FX-sensitized CAT assays in healthy subjects (Supplementary Figure 2) and in post-CABG surgery patients (Supplementary Figure 3). The ETP measured in the standard CAT assay is the most important influencer of the ETP of the FII sensitized assay. For the FV- and FX-sensitized

CAT assays, the association with the standard CAT assay are less pronounced, as the effect of FV and FX within the normal range are usually not detected by standard CAT. On the contrary in post-CABG surgery patient samples, in which the levels of FII, FV, and FX are reduced, the role of FV in the standard CAT assay becomes substantially larger, as indicated by the correlation of the peak height and ETP of the standard CAT TG and the FV-sensitized CAT TG.

Clinical validation in patients undergoing CABG surgery

The clinical applicability of the sensitized CAT assays was investigated in patients undergoing a CABG procedure. CABG surgery is known to have a profound effect on the hemostatic balance, as the procedure causes blood loss, consumption of coagulation factors, heparinization and the administration of blood products to support coagulation. In this study, blood samples were taken before the start of the surgery and immediately after surgery and heparin neutralization.

The FII, FV, and FX sensitized CAT assays were performed (Figures 3B,D,F, respectively). The FII and FX activity was significantly lower in pre-CABG patients compared to healthy subjects (-19%, $p < 0.0001$ and −21%, $p < 0.0001$, respectively), and 23 and 32% of the pre-surgery patients were outside the normal range, respectively. The FV activity did not differ between healthy subjects and pre-CABG patients, and all patients were within the normal range prior to the start of surgery. After surgery, the FII, FV and FX activity was severely reduced compared to the pre-surgery coagulation factor activity. Moreover, the majority of patients had FII and FX activities below the normal range, post-surgery, and FV levels were outside the normal range in approximately half of the patients.

We further investigated the association of FII, FV and FX sensitized CAT assay results with the parameters of the original CAT assay performed on the pre-CABG surgery patient samples (Figure 4B). Similar to the healthy subjects, pre-CABG patients showed a significant positive association of the FII activity with the ETP and peak height quantified by regular CAT, and a positive association of FX with the ETP, peak height and velocity index. Additionally, a high FV activity was associated with a significantly longer lag time and time-to-peak.

In Figure 5 we show the associations of the FII, FV and FX activity as measured by the sensitized CAT assays with peri-surgical blood loss. Pre-surgery FV activity measured by the FV sensitized CAT assay is significantly and positively associated with the post-surgery blood loss in the intensive care unit, and the total blood loss volume during and after the surgery. In contrast, FII and FX activity did not show a significant relationship with blood loss during or after surgery.

Discussion

The TG assay is used in research labs worldwide to study the coagulation potential in research settings. Over the last decades, many reports have shown the association of lower TG and bleeding (3, 4, 8). Vice versa, higher TG has been associated with a higher risk of thrombotic events, such as venous thromboembolism, stroke, and myocardial infarction (5, 7, 31). As a results, efforts have been made to bring the TG assay into the clinical laboratories, to support clinical decision making (17, 32–34).

The TG assay was originally designed as a global test of hemostasis, including most of the relevant coagulation factors present in the blood. Additional methods have been developed for the TG assay, to determine the role of platelet count and function by measuring TG in platelet rich plasma (35–37), and to determine the added effect of other blood cells and components by measuring TG in whole blood (38–40). Moreover, thrombomodulin can be added to the TG assay to investigate the function of the anticoagulant activated protein C pathway (41–43), which causes the increased risk of thrombosis in women using oral contraceptives (21, 44). Furthermore, the sensitization of the TG assay for the detection of functional levels of FVIII and FIX, respectively in hemophilia A and hemophilia B patients, leads to the more accurate prediction of bleeding risk in these patients (27).

In the current study, we investigated the potential of sensitizing the TG assay to the factors of the prothrombinase complex, which is pivotal in the production of thrombin during TG. The formation of the prothrombinase complex is considered one of the pivotal steps in the hemostatic system. Homozygous deficiency of prothrombinase cofactor FV is rare and is associated with mild to severe hemorrhagic symptoms (45), whereas FX deficiency is one of the most severe rare coagulation defects, causing bleeding symptoms such as hematomas, and umbilical cord, gastrointestinal, and central nervous system bleeding (46). Moreover, prothrombin is essential to the formation of active thrombin, and the therapeutic lowering of its plasma level has been shown to be associated with bleeding episodes in multiple patient groups. We have previously shown that prothrombin and FX, and to a lesser extent FV, are important influencers of TG parameters (19). The current study shows that FII, FV, and FX correlate with the ETP and peak height, which is in line with previous findings that the ETP and peak height are most sensitive to variations of coagulation factor levels in plasma (47). Nevertheless, a reduction in the original TG peak height or ETP can be caused by a reduction of prothrombin, FV or FX, but also a reduction of other coagulation factors, such as FVIII or FIX (48), an increase of antithrombin levels (19), the use of anticoagulants (49) or other changes in the hemostatic system. In this study we set out to develop TG-based assays that can pinpoint changes in the function of FII, FV, and FX. Our

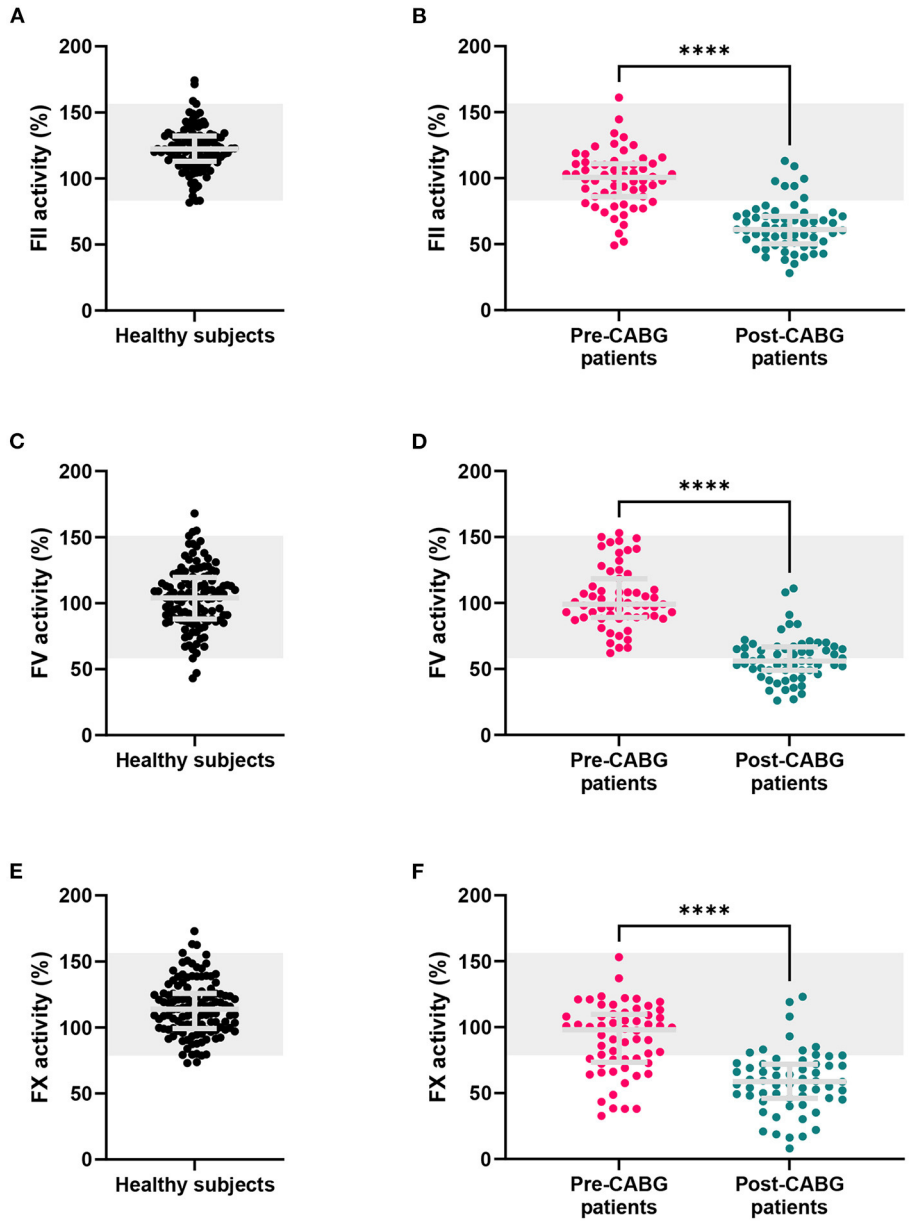

FIGURE 3
FII, FV and FX sensitized CAT assay results in healthy subjects, and patients before and after CABG surgery. **(A,B)** FII sensitized CAT assay results, **(C,D)** FV sensitized CAT results and **(E,F)** FX sensitized CAT were measured in healthy subjects and coronary artery bypass graft (CABG) patients before and after surgery. Results are depicted as individual data points with the median and inter-quartile range. The reference range for healthy individuals is marked in gray. ****$p < 0.0001$.

results indicate that the developed TG assays have a high level of robustness to measure the activity of FII, FV, and FX. Furthermore, because this methodology is based on the original CAT assay, it is possible to use the assay in all labs that currently use the TG assay, in a similar fashion as the assessment of TM and APC sensitivity in the CAT assay. In addition, this assay can be performed alongside the routine CAT measurement, as the measurement conditions are the same, and only a small additional sample volume is required. Moreover, the development of these sensitized CAT assays can serve as an example to create other CAT assays sensitized to for example FVII, or anticoagulant factors such as antithrombin.

We further studied the clinical applicability of the newly developed assays in patients undergoing coronary artery bypass graft (CABG) surgery. Patients undergoing CABG surgery are known to be at risk for both bleeding and thrombosis due to

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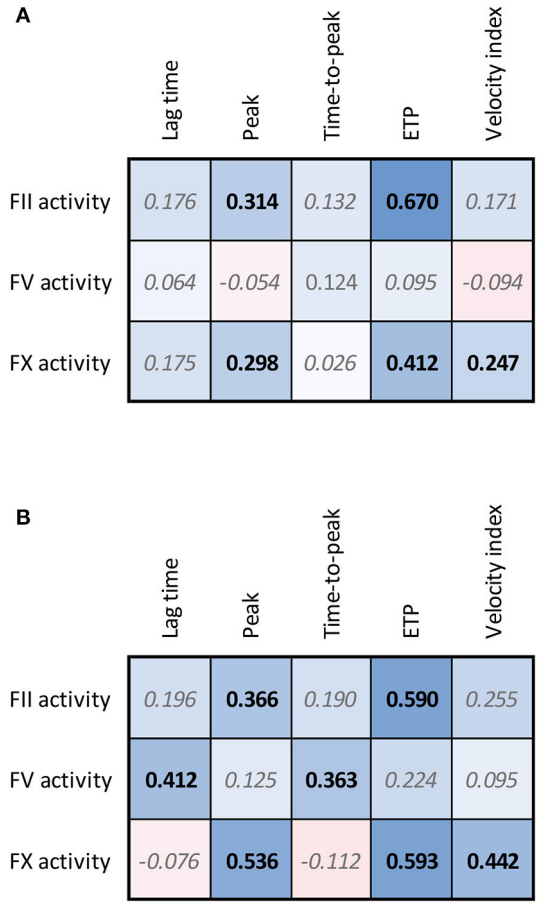

FIGURE 4
Correlation matrices for FII, FV, and FX sensitized CAT data and regular TG parameters in healthy subjects **(A)** and patients undergoing CABG surgery **(B).** The spearman correlation coefficient is shown in the confusion matrix, and the color of the cells shows the strength of the association. Significant associations are shown in black font ($p < 0.05$).

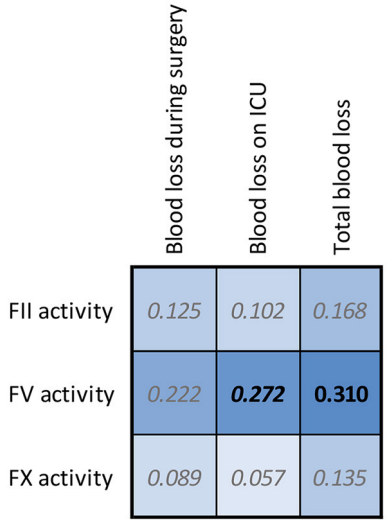

FIGURE 5
Correlation matrices for FII, FV, and FX sensitized CAT results in CABG patients and the blood loss during and after surgery. The spearman correlation coefficient is shown in the confusion matrix, and the color of the cells show the strength of the association. Significant associations are shown in black font ($p < 0.05$).

an imbalance of coagulation factors caused by heparinization before the surgery, hemodilution during the surgery and protamine administration (50). We previously found that TG was lower in patients undergoing CABG surgery pre-surgery compared to healthy subjects (51), and the CABG surgery, as expected, lead to an even more pronounced difference in TG potential. Therefore, we quantified these changes further by applying the FII, FV, and FX sensitized TG assays.

Our results show that FII, FV, and FX are severely reduced after CABG surgery, reaching levels of 48%, 44%, and 49% of the original plasma levels, respectively. These findings are in agreement with the report of Coakley et al. describing similar decreases of FII, FV, and FX (52). Reports in an earlier cohort of patients undergoing surgery with cardiopulmonary bypass (CPB), revealed that the reduction of TG in CPB patients was mainly attributable to a reduction of prothrombin conversion (50). This indeed is in line with the current finding that all components of the prothrombinase complex FII, FV and FX are severely reduced in patients after CABG surgery. Moreover, we investigated whether pre-surgical FII, FV and FX activity in the sensitized CAT assay is associated with the amount of blood loss during the surgery and found that high FV activity is associated with significantly higher blood loss. Although this seems counterintuitive, the dual role of FV in the coagulation system has been described in the past (53). Correlations between changes in coagulation factor activity that are associated with an increased risk of bleeding are of clinical importance if pre-surgery coagulation factor activities are indicative of bleeding during surgery. Especially the combination of changes of several coagulation factor activity levels or hemostatic parameters resulting in an specific pro- or anticoagulant hemostatic profile could be of interest in the prediction of bleeding complications on an individual basis.

In conclusion, we have developed CAT-based assays that are specifically sensitized to the action of FII, FV and FX in the TG process. The assay was shown to be reproducible and experimental variation was low. Moreover, the assays were able to pinpoint the changes that occur during and after CABG surgery to the prothrombin conversion pathway, and more specifically to the reduced contribution of FII, FV and FX.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Review Ethics Committee of Maastricht University Medical Center. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CB, RL-K, and BL conceived and designed the analysis. CB and JK collected the data. CB, ML, RL-K, and BL contributed data or analysis tools. CB, MN, and RL-K performed the analysis. CB, MN, RL-K, and BL prepared the first draft of the paper and the other authors revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

CB, JK, MN, RL-K, and BL are employees of Synapse Research Institute.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1000812/full#supplementary-material

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Investigation of thrombin generation assay to predict vaso-occlusive crisis in adulthood with sickle cell disease

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Introduction: Sickle cell disease (SCD) is an inherited hemoglobinopathy disorder. The main consequence is synthesis of hemoglobin S leading to chronic hemolysis associated with morbidity. The aim of this study was to investigate Thrombin Generation Assay (TGA) to assess hypercoagulability in SCD and TGA parameters as biomarkers of vaso-occlusive crisis (VOC) risk and hospitalization within 1 year.

Materials and methods: We performed TGA in platelet poor plasma (PPP) with 1 pM of tissue factor and 4 μM of phospholipid-standardized concentration, in duplicate for patients and controls. We measured thrombomodulin (TM), soluble endothelial Protein C Receptor and Tissue Factor Pathway Inhibitor (TFPI).

Results: A total of 113 adult patients with SCD, 83 at steady state and 30 during VOC, and 25 healthy controls matched on age and gender were included. Among the 83 patients at steady state, (36 S/S-1 S/β^0, 20 S/S$\alpha^{3.7}$, and 19 S/C-7 S/β^+) 28 developed a VOC within 1 year (median: 4 months [2.25–6]). We observed an increase of peak and velocity associated with a shortening of lagtime and time to peak (TTP) and no difference of endogenous thrombin potential (ETP) in patients compared to controls. TFPI ($p < 0.001$) and TM ($p = 0.006$) were significantly decreased. TGA confirmed hypercoagulability in all SCD genotypes and clinical status. The association of ETP > 1,207 nM.min and peak >228.5 nM presented a sensitivity of 73.5% and a specificity of 93.9% to predict VOC development within 1 year.

Conclusion: We have demonstrated a hypercoagulable state in SCD associated with chronic hemolysis. These preliminary findings suggest that TGA parameters, as ETP and peak, could be used to predict VOC development within 1 year.

KEYWORDS

thrombin generation assay, hypercoagulability, hemoglobinopathy, sickle cell disease, vaso-occlusive crisis

Introduction

Sickle cell disease (SCD) is an inherited hemoglobinopathy disorder caused by mutations in *HBB* gene with amino-acid substitution on β globin chain, leading to the production of the abnormal hemoglobin S (HbS). HbS polymerization in deoxygenated condition induces chronic hemolytic anemia and vaso-occlusive crisis (VOC), associating frequent hospitalization, morbidity and mortality caused by organ failure like stroke, acute chest syndrome (ACS), osteonecrosis, leg ulcers, retinopathy, pulmonary hypertension, priapism, and nephropathy (1). Moreover, a hypercoagulable state is reported in SCD with increased venous thromboembolism (VTE) or pulmonary embolism (PE) (1–3).

The pathophysiology of SCD is described as acquired thrombophilia with a complex mechanism (4). Hemostatic abnormalities as elevated prothrombin 1 + 2 fragment, D-dimer, factor VIII (FVIII), von Willebrand factor (vWf), tissue factor (TF) and decreased natural anticoagulants (protein C and S) or ADAMTS13 activity were reported (1, 2, 4–7). However, few studies have evaluated tissue factor pathway inhibitor (TFPI) and thrombomodulin (TM) in SCD. Other processes are involved in this hypercoagulable state, in particular, exposure to phosphatidylserine microvesicles from erythrocytes, monocytes and platelets (8–10), and formation of neutrophil extracellular traps (NETs) (11). This hypercoagulable state amplifies red blood cell (RBC) aggregation/adhesion (12), oxidative stress, inflammation and endothelial injury. A hypothesis of VOC amplification is thromboinflammation, induced by hemolysis and RBC adhesion (1, 5, 13, 14).

Thrombin generation assay (TGA) has demonstrated its relevance in thrombotic risk assessment in hereditary or acquired thrombophilia (15–18). TGA is a global assay which allows to differentiate hypo- or hyper-coagulable profiles (19, 20). Several studies have been published on TGA *ex vivo* in patients with SCD in steady state but the results are inconsistent requiring further studies (21–23).

The aim of this study was to evaluate the use of TGA to assess hypercoagulable state in SCD, and to investigate whether TGA parameters could be used to predict VOC and hospitalization within a year.

Materials and methods

Study design, patients, and controls

All patients in the study were diagnosed and treated for SCD at Rouen University Hospital between September 2018 and June 2021. Patients were included during an annual visit in our tertiary center (24). Patients with VOC were included less than 24 h after admission to emergency department. All patients treated with hydroxyurea had been treated for at least 3 years. All patients received a systematic annual visit to determine VOC development within a year. Patients were analyzed in four subgroups based on genotype and clinical status:

- Homozygous SCD (S/S) or β^0 thalassemia (S/β^0) at steady state;
- Homozygous SCD with $\alpha^{3.7}$ thalassemia (S/S$\alpha^{3.7}$) at steady state;
- Heterozygous SCD with C hemoglobin (S/C) or β^+ thalassemia (S/β^+) at steady state;
- Patients hospitalized for VOC with any genotype.

Prospective data were collected and completed from medical records. Clinical data included age, sex, and history of thrombosis. Patients on long-term anticoagulant therapy, pregnant women, patients aged less than 18 years, and patients with confirmed coagulation factor deficiency were excluded.

Blood samples were obtained from 25 healthy controls matched on age and gender, who had no history of bleeding, no thrombosis, no factor deficiency (evaluated with coagulation factor measurement), and no inherited thrombophilia (Factor V Leiden mutation, Factor II Leiden mutation, antithrombin, protein S and protein C deficiency). Informed consent was obtained from all subjects.

The study was performed in accordance with the Declaration of Helsinki on biomedical research involving human subjects. The study was approved by the institutional review board (Rouen University Hospital

TABLE 1 Characteristics of study population.

	Controls ($n = 25$)	S/S-Sβ ^{0} ($n = 37$)	S/Sα $^{3.7}$ ($n = 20$)	S/C-Sβ $^+$ ($n = 26$)	VOC ($n = 30$)
Clinical characteristics					
Age (years)	38.2 ± 11.8	31.1 ± 11.4	38.4 ± 4.9	37.1 ± 14.8	31.0 ± 9.2
Male *n* (%)	11 (44)	13 (35)	11 (55)	12 (46.1)	15 (50)
Hydroxyurea *n* (%)		31 (83.7)[a]**	16 (80)[b]**	6 (23)	20 (66.6)[c]*
Osteonecrosis *n* (%)		8 (21.6)	9 (45)[b]*	4 (15.3)	3 (10)
Retinopathy *n* (%)		2 (5.4)[a]*	6 (30)	8 (30.7)	11 (36.6)
Vasculopathy *n* (%)		6 (16.2)	0 (0)	2 (7.7)	5 (16.6)
ACS *n* (%)		16 (43.2)	7 (35)[b]*	2 (7.7)	8 (26.7)
Cholecystectomy *n* (%)		8 (21.6)	10 (50)	5 (19.2)	6 (20)
Splenectomy *n* (%)		0 (0)	1 (5)	2 (7.7)	4 (13.3)
PE/VTE *n* (%)		5 (13.5)	3 (0.15)	1 (3.8)	0 (0)
Coagulation tests					
PT (sec)		14.2 [13.2–15.6]	14.3 [13.1–15.5]	14.6 [13.4–15.2]	15.1 [14.5–15.7]
aPTT (sec)		34.3 [32.9–38.1]	35.3 [31.1–39.1]	35.1 [31.9–36.7]	34.5 [33.2–36.4]
Fibrinogen (g/L)		2.75 [2.26–3.36]	2.90 [2.10–3.20]	2.56 [2.24–2.83]	3.09 [2.74–3.52][c]*
Hematological parameters					
RBCs (T/L)		2.85 [2.35–3.42][a]**	3.04 [2.83–3.57][b]*	4.02 [3.78–4.64]	2.79 [2.42–3.61][c]**
Hemoglobin (g/dL)		8.5 [7.8–10.2][a]*	8.5 [7.9–9.2][b]**	10.5 [10.1–11.3]	8.8 [7.9–10.2][c]*
Hematocrit (%)		0.24 [0.22–0.29][a]*	0.25 [0.23–0.28][b]*	0.30 [0.29–0.32]	0.25 [0.23–0.29][c]*
MCV (fL)		88.4 [79.5–105.3][a]**	83.4 [76.1–86.6][b]*	73.5 [68.6–78.6]	90.5 [79.8–99.7][c]**
MCHC (g/dL)		34.9 [32.7–35.8]	33.4 [32.7–33.9][b]*	35.9 [33.7–35.9]	34.9 [33.9–35.8][d]*
Platelets (G/L)		365 [244–439][a]**	326 [216–390][b]*	186 [159–261]	307 [247–410]
Leukocytes (G/L)		8.3 [7.1–10.2][a]*	7.0 [5.0–9.4]	6.0 [4.6–6.4]	10.3 [7.7–12.1][c]**
Neutrophils (G/L)		4.89 [2.98–6.28]	3.86 [2.76–5.04]	3.10 [2.63–3.60]	5.59 [3.99–7.31][c]*
Lymphocytes (G/L)		2.67 [1.77–3.40]	1.84 [1.34–2.79]	1.76 [1.31–2.70]	2.57 [1.93–3.58][c]*
Monocytes (G/L)		0.92 [0.57–1.19][a]**	0.69 [0.53–0.94]	0.45 [0.32–0.62]	0.88 [0.54–1.29][c]**
Hemolysis parameters					
Reticulocytes (G/L)		251.7 [155.5–322.7][a]**	250.1 [191.1–295.6][b]*	138.0 [108.7–150.1]	275.9 [180.8–384.9][c]**
LDH (IU/L)		410 [364–517][a]**	385 [282–578][b]*	225 [185–294]	418 [354–497][c]**
Indirect bilirubin (μmol/L)		25.0 [19.0–37.0]	23.0 [18.0–35.0]	18.0 [15.0–26.0]	29.0 [19.5–45.5]
Hemoglobin profile					
HbS (%)		83.6 [65.3–91.05][a]*	87.2 [62–91.38][b]*	47.7 [46.6–52.7]	81.9 [48.6–87.5]

Data are expressed as median ± [IQR] except for age (mean ± SD) and clinical characteristics, *n* is the total number of patients (%).
ACS, acute chest syndrome; PE/VTE, pulmonary embolism/venous thromboembolism; PT, prothrombin time; aPTT, activated partial thromboplastin time; RBC, red blood cells; MCV, mean corpuscular volume, MCHC, mean corpuscular hemoglobin concentration; LDH, lactate deshydrogenase; HbS, hemoglobin S.
[a]Indicates a significant difference between S/S-S/β^0 and S/C-S/β $^+$.
[b]Indicates a significant difference between S/Sα$^{3.7}$ and S/C-S/β $^+$.
[c]Indicates a significant difference between VOC and S/C-S/β $^+$.
[d]Indicates a significant difference between S/Sα$^{3.7}$ and VOC.
*$p < 0.05$.
**$p < 0.001$.

Authorization protocol number: E2021-78) and is declared in clinical trials (clinical trials registration number: NCT05376046).

Sample collection

Platelet poor plasma (PPP) samples were obtained from the initial blood test, taken by antecubital venipuncture with a 21-gauge needle and collected in vacutainer tubes containing buffered 0.109 M trisodium citrate (Greiner) (1 part of citrate 3.2%/nine parts of blood). PPP was prepared 1 h after sampling, by double centrifugation of citrated blood for 15 min at 2,250 g. PPP was stored in aliquots at −80°C until analysis and run within 4 months with prior thawing in water at 37°C for 5 min.

Standard follow-up included dipotassium EDTA tubes (BD Vacutainer EDTA, Plymouth) for blood counts and plasma from lithium heparin tubes with gel separator (BD Vacutainer LH, Plymouth) for biochemical parameters.

TABLE 2 Correlation of TGA parameters in all SCD genotypes at steady state.

	Lagtime r (*p*-value)	ETP r (*p*-value)	Peak r (*p*-value)	Time to peak r (*p*-value)	Velocity r (*p*-value)
Fibrinogen (g/L)	0.12 (0.28)	**0.31 (0.003)**	**0.33 (0.002)**	−0.03 (0.81)	**0.3 (0.006)**
Leukocytes (G/L)	−0.04 (0.70)	0.12 (0.26)	**0.22 (0.04)**	−0.13 (0.24)	**0.22 (0.03)**
Monocytes (G/L)	−0.17 (0.10)	0.08 (0.44)	**0.32 (0.002)**	**−0.32 (0.003)**	**0.36 (0.0007)**
Reticulocytes (G/L)	−0.09 (0.39)	0.21 (0.07)	**0.32 (0.006)**	−0.15 (0.19)	**0.30 (0.0089)**
Platelets (G/L)	**−0.32 (0.002)**	0.06 (0.59)	0.03 (0.76)	**−0.39 (0.002)**	0.01 (0.88)
LDH (IU/L)	**−0.27 (0.03)**	0.06 (0.66)	**0.36 (0.005)**	0.35 (0.04)	**0.4 (0.002)**

Data are expressed as r Pearson correlation and (*p*-value). TGA, thrombin generation assay; ETP, endogenous thrombin potential. Bold values are for significant values.

TABLE 3 Comparison of TGA parameters between patients and controls.

TGA parameters	Controls (*n* = 25)	S/S-S/β ^{0} (*n* = 37)	S/Sα $^{3.7}$ (*n* = 20)	S/C-S/β $^{+}$ (*n* = 26)	VOC (*n* = 30)
Lagtime (min)	7.50 [6.67–7.93]	4.17 [3.75–4.58][a]**	4.37 [3.96–4.74][a]**	4.38 [3.96–4.84][a]**	4.58 [3.90–5.21][a]**
ETP (nM.min)	1184 [1101–1282]	1190 [1078–1301]	1114 [912.2–1300]	1212 [1090–1422]	1177 [1040–1380]
Peak (nM)	144.9 [119.1–153.0]	239.7 [196.3–278,1][a]**	208.8 [165.9–253.8][a]**	213.6 [173.5–266.9][a]**	225.2 [194.6–266.9][a]**
Time to peak (min)	12.71 [11.36–13.14]	7.08 [6.46–8.44][a]**	7.71 [7.13–8.12][a]**	7.50 [7.08–8.54][a]**	7.50 [6.67–8.44][a]**
Velocity (nM.min^{-1})	28.98 [21.24–33.18]	77.12 [57.31–107.3][a]**	64.57 [45.83–97.25][a]**	67.32 [49.18–92.08][a]**	71.42 [59.97–108.6][a]**

Data are expressed as median ± [IQR], *n* is the total number of patients. TGA, thrombin generation assay; VOC, vaso-occlusive crisis; ETP, endogenous thrombin potential.
[a]Indicates a significant difference with controls. **$p < 0.001$.

TABLE 4 Comparison of TGA parameters between steady state and VOC.

TGA parameters	Steady state (*n* = 83)	VOC (*n* = 30)	*P*-value
Lagtime (min)	4.17 [3.96–4.58]	4.58 [3.90–5.21]	0.11
ETP (nM.min)	1175 [1071–1319]	1177 [1040–1380]	0.65
Peak (nM)	221.3 [177.8–269.1]	225.2 [194.6–266.9]	0.66
Time to peak (min)	7.50 [6.67–8.12]	7.50 [6.67–8.43]	0.63
Velocity (nM.min^{-1})	68.9 [50.9– 100.4]	71.4 [59.9–108.6]	0.50

Data are expressed as median ± [IQR], *n* is the total number of patients. TGA, thrombin generation assay; VOC, vaso-occlusive crisis; ETP, endogenous thrombin potential.

Standard coagulation assays

Prothrombin time (PT) (Neoplastin, Diagnostica Stago, Asnières sur Seine, France) and activated partial thromboplastin time (aPTT) (PTT-A, Diagnostic Stago, Asnières sur Seine, France) were measured with STAR Max. Fibrinogen levels were measured in plasma by Clauss clotting method (STA-Liquid Fib, Diagnostica Stago, Asnières, France). All hemostasis tests were performed in citrated plasma samples.

Soluble thrombomodulin, soluble endothelial protein C receptor, and tissue factor pathway inhibitor

Human BDCA-3 (TM) (Quantikine®, Bio-Techne Brands, Abingdon, United Kingdom) and human soluble Endothelial Protein C Receptor (sEPCR) (DuoSet ELISA®, Bio-Techne Brands, Abingdon, United Kingdom) (FilterMax F3®, Molecular Devices, San Jose, CA, United States) were measured at 450 nm. TFPI was measured by immuno-enzymatic kit (Asserachrom® Total TFPI, Diagnostica Stago, Asnières, France).

Thrombin generation assay

Thrombin generation assay was performed in PPP in duplicate with 1 pM of tissue factor and 4 μM of phospholipid (PPP low reagent, Diagnostica Stago, Asnières, France). TGA was measured by Calibrated Automated Thrombography (CAT), Fluoroscan Ascent Fluorometer (Thermo Scientific Lab Systems®, Helsinki, Finland) and Thrombinoscope® software (Thrombinoscope 5.0 BV®, Maastricht, Netherlands). We used 96-well plates (Immunlon®2HB, Thermo Scientific, Rochester, NY, USA). The TGA parameters of thrombin generation curve were considered: lagtime (corresponding to the first trace of thrombin formation), time to peak (TTP; time necessary for thrombin maximal value), peak (maximal thrombin concentration), endogenous thrombin

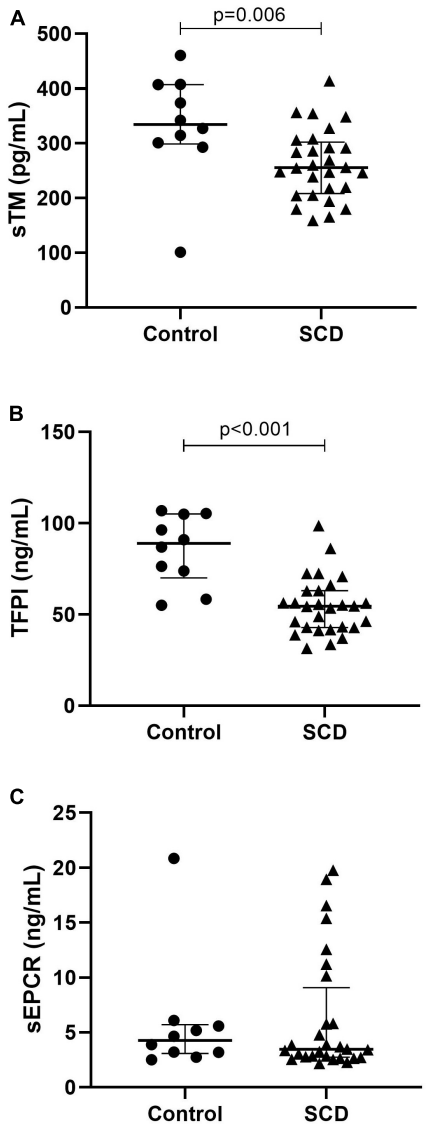

FIGURE 1
Anticoagulant protein expression in sickle cell disease. With thrombomodulin **(A)**, tissue factor pathway inhibitor **(B)**, and soluble endothelial protein C receptor **(C)**. TFPI, tissue factor pathway inhibitor; sEPCR, soluble endothelial protein C receptor, TM, thrombomodulin; SCD, sickle cell disease. Data are expressed as median [IQR]. *P*-values comparing control group and SCD patients in steady state are from Mann–Whitney test.

potential (ETP; area under the thrombin time concentration curve), and velocity [calculated: peak/(TTP-lagtime)]. TGA was carried out according to International Society of Thrombosis and Hemostasis (ISTH) recommendations (25). The coefficients of variation (CV) for intra-assay and inter-assay were calculated with pooled normalized plasma. CV for intra-assay and inter-assay were, respectively, 1.7 and 9.2% for lagtime, 1.7 and 8.6% for TTP, 2.5 and 6.8% for ETP, 4.7 and 6.8% for peak, 9.4 and 8.8% for velocity. Experiments were done in duplicate for controls and patients. No result could be validated if the agreement between two wells had a variation of >10%.

Hemolysis parameters

Hemoglobin, reticulocyte count (RET) and plasma lactate dehydrogenase levels (LDH) were measured in samples collected for routine follow-up, in parallel with those collected for thrombin generation. Blood counts were measured on XN-9000 (Sysmex, Villepinte, France). LDH and indirect bilirubin levels were determined on cobas® 8000 chemistry analyzer (Roche Diagnostics, Mannheim, Germany).

Other laboratory parameters

Hemoglobin profile was determined by high performance liquid chromatography (HPLC) (Variant II Biorad, CA, United States), by capillary electrophoresis on Capillarys 3 Octa® (Kit hydragel hémoglobineSebia, Lisses, France) and iso-electrofocalisation. The presence of $\alpha^{3.7}$ thalassemia was determined using a single-tube, multiplex-PCR assay.

Statistical analysis

Data are expressed as medians and interquartile ranges (IQR). Statistical analyses were performed with GraphPad Prism for Windows, version 9.2 (GraphPad Software, San Diego, CA, United States). Pearson's correlation was used to determine the correlation between two variables. TGA parameters between patients and controls were compared using a Kruskall–Wallis ANOVA with Dunn's multiple comparisons post-test or Mann–Whitney test. Receiver operating characteristic (ROC) curves were built for significant clinical characteristics. *P*-values < 0.05 were considered to be statistically significant.

Results

Demographic characteristics

A total of 113 patients with SCD were included in this study, 83 at steady state and 30 during VOC. Among the 83 patients at steady state 37 were S/S-S/β^0, 20 were S/S$\alpha^{3.7}$, and 26 were S/C-S/β^+. Patient characteristics are presented in **Table 1**. Among the 83 patients at steady state, 28 developed a VOC within 1 year (median: 4 months [2.25–6.0]). Seventy-three were treated with hydroxyurea.

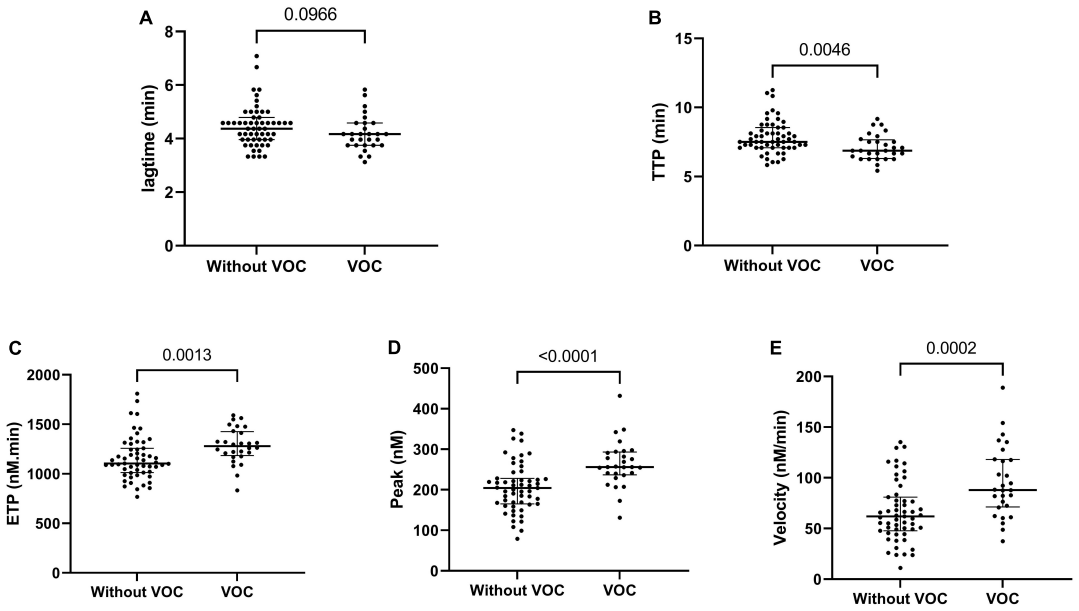

FIGURE 2
Thrombin generation parameters associated with VOC development in the year following steady state. Lagtime **(A)**, time to peak **(B)**, endogenous thrombin potential **(C)**, peak thrombin **(D)**, and velocity **(E)**. Data are expressed as median [IQR]. P-values comparing SCD in steady state and SCD developing VOC in a year are from Kruskall–Wallis ANOVA with Dunn's multiple comparisons post-test.

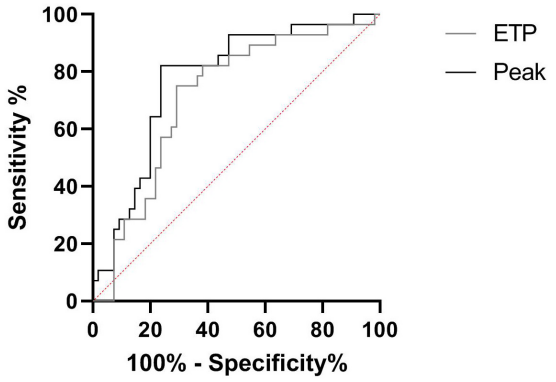

FIGURE 3
Receiver operating characteristic (ROC) curve of VOC prediction by TGA. Comparison between SCD in steady state and SCD developing VOC in a year. ETP, endogenous thrombin potential (with an ETP > 1,207 nM.min: AUC: 0.71, sensitivity: 75.1%, specificity: 70.9%; With a Peak >228.5 nM: AUC: 0.77, sensitivity: 82.1%, specificity: 76.4%).

Thrombin generation assay

We evaluated the association between TGA parameters, hemolysis markers (hemoglobin, RET count, LDH, indirect bilirubin), fibrinogen and blood counts in SCD at steady state (Table 2).

At steady state, we observed a correlation between peak and velocity, in particular, and fibrinogen, leukocytes, monocytes, platelets, RET and LDH. TGA association in the 4 subgroups (S/S-S/β^0, S/S$\alpha^{3.7}$, S/C-S/β^+, and VOC) is shown in Supplementary Tables 1–4.

TGA revealed hypercoagulability in patients compared to controls. Lagtime and TTP were significantly lower and associated with increased peak and velocity in all 4 SCD subgroups compared to controls (Table 3). No differences were observed in ETP in S/S-S/β^0 (p = 0.96), S/S$\alpha^{3.7}$ (p = 0.27), S/C-S/β^+ (p = 0.45), and VOC (p = 0.73). No differences were observed in TGA parameters between S/S-S/β^0, S/S$\alpha^{3.7}$, S/C-S/β^+, and VOC.

Then, we pooled the three genotype subgroups at steady state (i.e., S/S-S/β^0, S/S$\alpha^{3.7}$, and S/C-S/β^+, n = 83) for comparison with the VOC subgroup including all genotypes (Table 4). No differences were observed in thrombin generation parameters between steady state and VOC.

Physiological anticoagulant proteins

We compared TM, TFPI and soluble endothelial protein C receptor (sEPCR) between patients and controls at steady state (Figure 1). Median TM was significantly decreased in patients (255.5 pg/mL, [208.2–302.3]) compared to controls (334.5 pg/mL, [298.9–407.2], p = 0.0061). Median TFPI level was significantly decreased in patients (54.54 ng/mL [42.89–63.05])

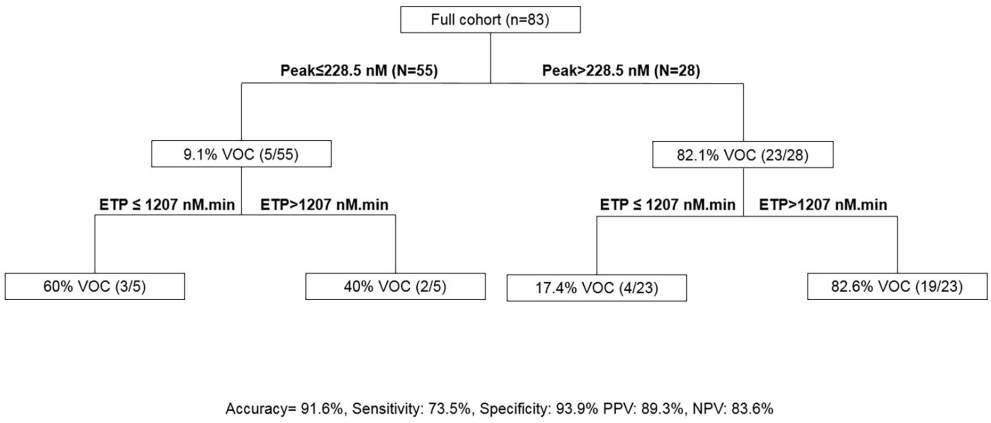

FIGURE 4
Calibrated automated thrombography (CAT)-based algorithm to predict VOC. ETP, endogenous thrombin potential; PPV, positive predictive value; NPV, negative predictive value, VOC, vaso-occlusive crisis.

compared to controls (88.97 ng/mL [69.99–105.1], $p < 0.0001$). Median sEPCR was not different between patients and controls.

We determined differences in TFPI, TM, and sEPCR between the three genotype subgroups at steady state. TFPI was significantly decreased in S/S-S/ß^0 (46.03 ng/mL, [40.03–59.35]) compared to S/Sα$^{3.7}$ (66.12 ng/mL, [58.78–78.47], $p = 0.046$) but not with S/C-S/ß$^+$ (54.80 ng/mL [43.18–56.35], $p = 0.37$). TM was decreased in S/S-S/ß^0 (219.7 pg/mL [179.7–251.0]) compared to S/Sα$^{3.7}$ (307.9 pg/mL [264.6–352.2], $p < 0.001$) and S/C-S/ß$^+$ ($n = 10$) (288.7 pg/mL [240.4–334.0], $p = 0.0052$). No difference in sEPCR was observed.

Vaso-occlusive crisis prediction by thrombin generation assay

We prospectively monitored patients at steady state to determine which of them would be hospitalized for VOC ($n = 28$) within 1 year. Patients with VOC development had decreased TTP and increased ETP, peak and velocity at steady state, compared to patients without VOC development (**Figure 2**). The risk to develop VOC, determined with a ROC curve, was an ETP of >1,207 nM.min and a peak of >228.5 nM (AUC: 0.71, sensitivity: 75.1%, specificity: 70.9%; AUC: 0.77, sensitivity: 82.1%, specificity: 76.4%, respectively) (**Figure 3**).

The association of an ETP of >1,207 nM.min and a peak of >228.5 nM presented a sensitivity of 73.5% and a specificity of 93.9% to predict VOC in SCD (**Figure 4**).

Discussion

Our study aimed to evaluate the use of TGA to assess hypercoagulable state in SCD, and to investigate whether TGA parameters could be used to predict VOC development within 1 year. Our results demonstrate a hypercoagulable state in SCD and a good correlation between ETP-peak and VOC risk of hospitalization within 1 year.

Sickle cell disease has been described as inherited thrombophilia caused by a complex pathophysiology, in particular, chronic hemolysis. VOC are characterized by hemolytic anemia, endothelial damage, and potentially life-threatening complications (26). In addition to hemolysis, acquired hypercoagulability induced several hemostatic changes like TF and phospholipid overexpression, endothelial dysfunction and anticoagulant pathways, with decreased protein S and protein C (5).

Venous thromboembolism is common in adults with SCD and was found in 18.8% of SCD patients (27). We demonstrated a hypercoagulable state on TGA with a significantly shortened lagtime and TTP associated with a higher peak and velocity in all genotypes at steady state and during VOC. Surprisingly, ETP was similar in all four SCD subgroups and the control group. TGA parameters were poorly correlated with hemolysis markers (RETs and LDH) and with leukocytes and monocytes, confirming a complex pathophysiology and a thromboinflammatory hypothesis. However, we were not able to demonstrate a difference between patients at steady state or during VOC. Anticoagulant pathways were previously described with decreased antithrombin, protein S and protein C (4, 28). We demonstrated significantly decreased plasma levels of total TFPI and TM between patients compared to controls. Based on these results, we hypothesize that anticoagulant pathways are chronically consumed in SCD caused by vascular hemolysis and limited ETP (29). Moreover, we observed a non-significant increase of SCD patients with a history of thromboembolism. Our non-significant results are probably secondary to the small number of events.

Several studies have evaluated TGA in SCD. TGA was studied for the first time by Betal et al. in 23 patients with S/S-S/β^0. These authors reported a significantly lower lagtime, ETP and TTP with no difference in peak (29). Conversely, Shah et al., with the same TGA protocol, reported a significant increase of ETP ($p < 0.01$), peak ($p < 0.01$), and D-dimer ($p < 0.05$) during crisis associated with a lower lagtime ($p < 0.01$) and velocity ($p < 0.01$) in paired adults (21). Gerotziafas et *al* demonstrated no difference in ETP and lagtime associated with a higher peak and velocity and a lower TTP in 92 SCD patients at steady state treated or not with hydroxyurea using 5 pM of TF and 4 μM of PL concentrations (22). Moreover, previous studies demonstrated a lower ETP compared to healthy controls, in SCD patients treated with hydroxyurea and exchange blood transfusion (23, 30). A lack of standardization in performing the assays contributed largely to a poor correlation between assays and study results with TGA (31).

Several studies have demonstrated thrombin generation modification in SCD treated with hydroxyurea (22, 23). Hydroxyurea reduced hypercoagulability in treated patients with a higher lagtime and TTP, a lower peak and velocity and no difference in ETP compared to untreated patients. Only one study revealed no difference between patients treated or not with hydroxyurea with TGA (30). In our study, we did not compare patients treated or not with hydroxyurea because a significant proportion of homozygous patients were treated long-term. Six heterozygous SCD patients were treated according to recommendations for this therapy in patients who reported vasculopathy, ACS or more that 3 VOCs per year (32).

The ability to predict the phenotype of an individual with SCD could guide therapeutic decision making. An interesting result of this study was the use of TGA to predict VOC. Increased ETP and peak were associated with VOC development requiring hospitalization within a year. Moreover, the association of an ETP of >1,207 nM.min and a peak of >228.5 nM presented a sensitivity of 73.5% and a specificity of 93.9% to predict VOC development during the year following the visit. Other scores to predict VOC severity in ACS are emerging (33). New therapeutics are used in SCD to prevent VOC like crizanlizumab, a monoclonal antibody targeted to P selectin (34). Our TGA results could be used to study therapeutics with the objective of preventing VOC.

Anti-platelet and anticoagulant therapies have been investigated in SCD with promising results in pre-clinical studies, but these results were not confirmed in clinical trials (2, 4, 14, 28, 35). In fact, adults with SCD are prone to develop hemorrhagic stroke (2). Recently, contact pathway inhibition was evaluated in mice and may provide a target to reduce hypercoagulable state in SCD (36).

The main limitation of our study is the sample size, with subgroups based on genotype or clinical phenotype. However, these preliminary findings require further exploration in a larger cohort comparing steady state and VOC in paired patients. A second limitation is the diversity of reagents or conditions (PPP, PRP, whole blood) for TGA in current clinical practice which may lead to different results. Moreover, we did not use an inhibitor of contact phase [corn trypsin inhibitor (CTI)], which may have an impact on TG parameters when using 1 pM TF (37). A comparison between PPP with and without CTI or TM would have been interesting to complete our TGA results. Recently, studies showed that TGA with TM was more sensitive to evaluate endothelial dysfunction (17, 18, 38). However, we followed ISTH recommendations to limit the impact (16, 25). Moreover, a new generation of CAT (ST Genesia) could be more standardized and facilitate VOC prediction (38). Finally, we did not explore all endogenous inhibitors of coagulation because lower AT, protein S and C were described. Betal and al hypothesized an upregulation of TFPI in SCD. Here we provide new data on total TFPI, TM, and sEPCR.

Conclusion

In this study, we have demonstrated, in all SCD genotypes, that a hypercoagulable state is associated with chronic hemolysis. Based on these preliminary findings, ETP and peak could be used to predict VOC development within 1 year.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the institutional review board (Rouen University Hospital) approved the study (Authorization protocol number: E2021-78). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

GF and FK performed the analysis and wrote the manuscript. MG, YB, AL, and CD included patients and critically revised the manuscript and results. VB critically revised the manuscript and results. VL discussed the obtained results and critically revised the manuscript. PB designed the research, analyzed, interpreted the data, and wrote the

manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.883812/full#supplementary-material

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Serial thrombin generation and exploration of alternative anticoagulants in critically ill COVID-19 patients: Observations from Maastricht Intensive Care COVID Cohort

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Background: COVID-19 associated coagulopathy (CAC) is associated with an increase in thromboembolic events. Current guidelines recommend prophylactic heparins in the management of CAC. However, the efficacy of this strategy in the intensive care population remains uncertain.

Objective: We aimed to measure thrombin generation (TG) to assess CAC in intensive care unit (ICU) patients receiving thromboprophylaxis with low molecular weight heparin (LMWH) or unfractionated heparin (UFH). In addition, we performed statistical modeling to link TG parameters to patient characteristics and clinical parameters. Lastly, we studied the potency of different anticoagulants as an alternative to LMWH treatment in *ex vivo* COVID-19 plasma.

Patients/Methods: We included 33 patients with confirmed COVID-19 admitted at the ICU. TG was measured at least twice over the course of 6 weeks after admission. Thrombin generation parameters peak height and endogenous thrombin potential (ETP) were compared to healthy controls. Results were subsequently correlated with a patient characteristics and laboratory measurements. *In vitro* spiking in TG with rivaroxaban, dabigatran, argatroban and orgaran was performed and compared to LMWH.

Results: Anti-Xa levels of all patients remained within the therapeutic range throughout follow-up. At baseline, the mean (SE) endogenous thrombin potential (ETP) was 1,727 (170) nM min and 1,620 (460) nM min for ellagic acid (EA) and tissue factor (TF), respectively. In line with this we found a mean (SE) peak height of 353 (45) nM and 264 (96) nM for EA and TF. Although fluctuating across the weeks of follow-up, TG parameters remained elevated despite thromboprophylaxis. *In vitro* comparison of LMWHs and direct thrombin inhibitors (e.g., agratroban, dabigatran) revealed a higher efficacy in reducing coagulation potential for direct thrombin inhibition in both ellagic acid (EA) and tissue factor (TF) triggered TG.

Conclusion: In a sub-group of mechanically ventilated, critically ill COVID-19 patients, despite apparent adequate anti-coagulation doses evaluated by anti-Xa levels, thrombin generation potential remained high during ICU admission independent of age, sex, body mass index, APACHE II score, cardiovascular disease, and smoking status. These observations could, only partially, be explained by (anti)coagulation and thrombosis, inflammation, and multi-organ failure. Our *in vitro* data suggested that direct thrombin inhibition compared with LMWH might offer an alternate, more effective anticoagulant strategy in COVID-19.

KEYWORDS

thrombin generation, COVID, anticoagulation, ICU, DOACs

Introduction

COVID-19 associated coagulopathy (CAC) has become increasingly evident with high rates of venous thromboembolism (VTE), particularly in severely affected patients (1–3). Therefore, national and international clinical guidelines advocated increased doses of low molecular weight heparin (LMWH) thromboprophylaxis in high-risk patients (4, 5). However, whether this strategy is effective enough in attenuating the hypercoagulable state and thus reducing risk of thrombosis remains unclear (6).

Both the clinical phenotype and the biochemical characteristics of CAC are markedly different from other coagulopathies (7–11). SARS-CoV-2 specific activated coagulopathy-related pathways interacting with host inflammation appear to be important drivers for CAC (9, 12). In addition to hypercoagulability, hypofibrinolysis also plays a role (7, 13–17), possibly explaining mixed results reported for associations between fibrinogen and d-dimer concentrations with thromboembolism (18–20). Furthermore, increased heparin resistance has been observed in severe COVID-19 disease (21–25).

Therefore, more insight into the underlying mechanisms of CAC by which heparins do (or do not) protect against thromboembolism is required. More insight will indicate alternative ways to improve thromboprophylaxis and clinical outcome in SARS-CoV-2 infected patients who experience a severe disease course.

In COVID-19 disease, increased values in the plasma thrombin generation have been associated with a worse prognosis (26, 27). Thrombin generation is a suitable tool to monitor coagulation potential in SARS-CoV-2 infection and has been assessed and adapted for CAC (28). Several reports suggest that thrombin generation is enhanced in CAC (17, 29, 30), independent of heparin treatment (17). However, other studies showed no differences in thrombin generation during various stages of disease severity in SARS-CoV-2 infection, suggesting coagulation exhaustion (31–33). In this paper the results of serial thrombin generation experiments, performed in a cohort of severely COVID-19 patients, admitted to the intensive care unit (ICU) for mechanical ventilation, are presented.

We hypothesized that thrombin generation is enhanced in subjects with CAC, despite treatment with unfractionated heparin (UFH) and LMWH (either prophylactic or therapeutic) and that alternative anticoagulants directed against thrombin or factor Xa might be more beneficial in attenuation of the hypercoagulable state in severe COVID-19 infection.

Methods

The manuscript was written following the STrengthening the Reporting of Observational studies in Epidemiology (STROBE) guidelines (34).

Study population

The Maastricht Intensive Care COVID (*MaastrICCht*) study is a prospective cohort study on patients with confirmed COVID-19 admitted to the ICU of the Maastricht University Medical Centre (MUMC+). The design has been described extensively elsewhere (35) and includes comprehensive serial hemostasis and coagulation phenotyping (13). The local institutional review board [Medisch Ethische Toetsingscomissie (METC) 2020-1565/300523] of the MUMC+ approved the study, which was performed based on the regulations of Helsinki. The study is registered in the Netherlands Trial Register (registration number NL8613).

This study included all participants with respiratory insufficiency requiring mechanical ventilation and at least one real-time polymerase chain reaction (RT-PCR) positive for SARS-CoV-2 RNA and a chest CT scan strongly suggestive for SARS-CoV-2 infection, based on a CORADS-score of 4–5 scored by a radiologist (36). Participants were followed until the primary outcome was reached (i.e., either died in the ICU or discharged from ICU). Every day, a comprehensive and uniform set of clinical, physiological, and laboratory variables was collected, reducing the chance of missing data. In addition, when patients were not available for blood withdrawal or laboratory testing failed, the measurement would be rescheduled for the next blood withdrawal.

Clinical, physiological variables

ICU care for COVID-19 was standardized as described extensively elsewhere (35). Medical history for cardiovascular disease (defined as: diabetes mellitus, myocardial infarction, hypertension, peripheral vascular disease) was scored at ICU admission. APACHE-II score on ICU admission and SOFA-score during ICU stay were calculated. Computed tomography pulmonary angiography (CTPA) was performed when pulmonary embolism was clinically suspected. Due to logistical reasons no routine compression ultrasonography for detection of deep venous thrombosis was performed as described (18).

Anticoagulation

During the early COVID-19 pandemic, incident thrombosis frequently appeared, resulting in new thromboprophylaxis recommendations. From March 25th 2020, at the start of the MaastrICCht cohort, the COVID specific thromboprophylaxis dosage in the ICU was Nadroparin 3,800, 5,700 and 7,600 IU for respectively <70, 70–90 and >90 kg. After the release of a new national guideline document on April 23rd 2020, this dose was increased (Nadroparin 5,700, 7,600 and 11,400 IU, respectively) (37). Patients who required therapeutic anticoagulants prior to hospital admission were started on therapeutic LMWH upon ICU admission. Vitamin K antagonists and direct oral anticoagulants (DOACs) were switched to therapeutic LMWH. Patients on extracorporeal membrane oxygenation (ECMO) or continuous renal replacement therapy (CRRT) received UFH, dosed on aPTT [heparin therapeutic range (HTR) 50–80s] and anti-Xa (HTR 0.3–0.7 IU/ml) guidelines (21).

Hemostasis sub-group

Ninety-four patients were enrolled from March 25th until June 12th in the MaastrICCht cohort. Enrollment for the hemostasis sub-group started later, on April 23rd 2020. During the first wave of COVID-19, 36 patients were included into the hemostasis sub-group. To align patients in their COVID-19 disease course, measurements from each patient were included from the day of intubation onwards. Timing from intubation allows for a fairer comparison between thrombin generation parameters and the disease course severity, where disease severity is defined as the need for mechanical ventilation on the ICU due to COVID-19. From April 23rd 2020 onwards additional thrombin generation assays were performed routinely twice weekly in all included MaastrICCht cohort patients. Patients who were in the ICU before April 23rd 2020 or who were transported from another hospital after intubation were also included, starting thrombin generation measurements from admission from April 23rd 2020 onwards. This means that some patients could be included in their first till the sixth week after intubation. This design has been described more extensively elsewhere (13).

Blood withdrawal and preparation

Daily arterial blood samples from all patients were collected from an arterial line in 7.2 mg K_2 EDTA (4.0 ml), serum and 3.2% (w/v) sodium citrate Vacutainer blood collection tubes (Becton Dickinson, Plymouth, UK). Fibrinogen concentrations were measured within 2 h of blood collection in citrated plasma, using a SysmexCS2100i hemostasis analyzer (Sysmex Corporation, Kobe, Hyogo, Japan). Detectable fibrinogen concentration had a maximum of 9 g/L. Concentrations of C-reactive protein (CRP, third generation, Roche Diagnostics, Basel, Switzerland) were measured on the COBAS®8000 by Roche Diagnostics in serum. Additional 3.2% (w/v) sodium citrate blood tubes were collected for thrombin generation measurements, twice a week for each patient. Platelet poor plasma (PPP) was obtained using two subsequent centrifugation steps: initial centrifugation of 2,490g for 5 mins, followed by 10,000g for 10 mins. Anti-Xa activity (biophen Heparin LRT; HYPHEN Biomed, Neuville-Sur-Oise, France) was measured

on a Sysmex CS2100i (Sysmex Corporation) in COVID-19 patient citrate plasma diluted 1:2 with reference pooled citrate plasma. Anti-Xa activity was determined using a LMWH calibration line (aXa-LMWH; HYPHEN Biomed). UFH activity was subsequently calculated with a previously determined formula: UFH anti-Xa = 1.55 * LMWH anti-Xa (21).

In the second part of the study a switch to *in vitro* experiments was made. For this purpose a separate COVID-19 pooled plasma was created from patients included in the overarching MaastrICCHt cohort. The pooled plasma had an average LWMH level of 0.3 U/ml was prepared by combining plasma of 40 patients with severe COVID-19 admitted to the ICU.

Thrombin generation

Thrombin generation is quantified by a thrombin generation curve over time and can be described by five parameters (38, 39). First, the endogenous thrombin potential is defined as the surface area under the thrombin generation curve indicating the total amount of thrombin generation that has been generated over the course of plasma coagulation. Second, the peak height (PH) of the thrombin generation curve indicates the maximal thrombin concentration reached. Lag time (start of the curve), velocity index (upward slope of the curve) and time to tail (end of the curve) are less used parameters (38, 39). Thrombin generation was performed in platelet-poor citrated plasma using the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, The Netherlands). Thrombin generation was optimized for COVID-19 as described extensively elsewhere (28). Briefly, thrombin generation was triggered by either 10 μg/ml ellagic acid (reflecting the intrinsic route of coagulation activation, similar to aPTT) or high tissue factor (reflecting the extrinsic route of coagulation activation, similar to PT) (PPP Reagent HIGH). Phospholipids were added in a 20/60/20 (PS/PC/PE) ratio to the concentration of 4 μM. Thrombin generation was assessed by adding a low-affinity fluorescent thrombin substrate (Z-Gly-Gly-Arg 7-amino-4-methylcoumarin) and utilizing a 390/460 nm filter. Machine setup and data recording were performed using the thrombinoscope software (Thrombinoscope BV, Maastricht, the Netherlands). *In vitro* spiking of normal pooled plasma and COVID-19 pooled plasma was performed by initial addition of 0.3 IU/ml fraxiparin (Mylan BV, Canonsburg, USA) and followed by further addition of 0.3–1.2 IU/ml fraxiparin (Mylan BV, Canonsburg, USA), 0–4 μg/ml argatroban (Goodlife pharma Nederland, Naarden, The Netherlands), 0–1.6 U/ml orgaran (Mylan BV, Canonsburg, USA), 0–320 ng/ml dabigatran (Boehringer Ingelheim bv, Ingelheim am Rhein, Germany) and 0–320 ng/ml rivaroxaban (Bayer BV, Leverkusen, Germany).

Statistical analyses

Data were analyzed using SPSS version 25. Data were included starting at the date of intubation until a maximum of 6 weeks of ICU admission. The first thrombin generation assay per week was included in the analyzes for each patient (if available). Admission characteristics were described using mean and standard deviation (SD), median and interquartile range or percentage, as appropriate. First, the mean anti-Xa level per week was investigated to detect the presence of LMWH or UFH. Second, the longitudinal trends in thrombin generation parameters were analyzed using linear mixed models, since these account for the interdependency in serial measurements over time within patients. First, in a crude model (model 1), we analyzed the association between time (week 1 to week 6) and thrombin generation parameters (endogenous thrombin generation potential and peak height). Time was treated as a categorical variable. Second, we adjusted thrombin generation data for age, sex, body mass index, APACHE II, cardiovascular disease, and smoking (model 2). Third, models 2 was additionally adjusted to investigate whether serial anticoagulation measurements (i.e., anti-Xa level and fibrinogen) (18), serial inflammation parameters (i.e., C-reactive protein) (9), serial multi-organ dysfunction parameters (40) or incident pulmonary embolism and deep venous thrombosis events could explain the association between time and thrombin generation parameters.

We report regression coefficients β with 95% confidence intervals (95%CI) and considered a p-value <0.05 statistically significant.

Results

Patient characteristics

We included 33 out of 36 patients from the COVID-19 hemostasis sub-group; from 3 patients no data on thrombin generation were collected. The mean ± SD age was 61.6 ± 9.7 years, 82% were men. The body mass index was 28.0 ± 4.3 kg/m^2. At ICU admission C-reactive protein was 202 ± 84 mg/L and fibrinogen was 6.6 ± 2.0 g/L. At ICU admission, the APACHE-II score was 16 ± 4, and the SOFA score was 7 ± 1.8. During ICU stay, 19 (58%) patients were diagnosed with pulmonary embolism, 1 patient was diagnosed with deep venous thrombosis (3%). Six patients (18.2%) received ECMO and seven patients (22.2%) received CRRT. The length of stay at the ICU was 33.4 ± 16.2 days, with a mortality rate of 36% (Table 1). Measured samples were included into the study based on weeks after ICU admission, weekly between 10 and 22 individual patient samples were included in the analysis, of which on average 46–92% of patients were treated with therapeutic doses

TABLE 1 Patient characteristics.

General	$n = 33$
Age, year, mean (SD)	61.6 (9.7)
Male, n (%)	27 (81.8)
Body mass index, kg/m^2, mean (SD)	28.0 (4.3)
Medical history	
Diabetes Mellitus type II, n (%)	1 (3%)
Hypertension, n (%)	9 (27.2%)
Malignancy, n (%)	4 (12.1%)
Myocardial infarction, n (%)	1 (3%)
Peripheral vascular disease, n (%)	1 (3%)
Smoker, n (%)	2 (6%)
Medication prior to admission	
Immunosuppression, n (%)	0 (0%)
Angiotensin II converting enzyme inhibitors, n (%)	5 (15.2%)
Angiotensin II receptor blocker, n (%)	4 (12.1%)
Calcium channel blockers, n (%)	1 (3%)
β-blockers, n (%)	4 (12.1%)
Diuretics, n (%)	2 (6%)
Lipid lowering agents, n (%)	5 (15.2)
Antiplatelet agents, n (%)	3 (9%)
Coumarins, n (%)	0 (0%)
Direct oral anticoagulants, n (%)	3 (9%)
ICU events	
SOFA score at admission, mean (SD)	7.0 (1.8)
APACHE II score at admission, mean (SD)	16.0 (4.4)
Length of ICU stay, days, mean (SD)	33.4 (16.2)
CRRT during ICU stay, n (%)	7 (21.2%)
ECMO during ICU stay, n (%)	6 (18.2%)
Thrombotic complications at ICU:	
Deep venous thrombosis, n (%)	1 (3%)
Pulmonary embolism, n (%)	19 (58%)
ICU mortality, n (%)	12 (36%)

SOFA-score, Sepsis-related Organ Failure Assessment; APACHE II score, acute physiology and chronic health evaluation; SAPS II, simplified acute physiology; CRRT, continuous renal replacement therapy; ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit.

of either LMWH or UFH (Table 2). Anti-Xa levels for the whole group were 0.56 ± 0.23 IU/ml.

Ellagic acid triggered thrombin generation

The mean (SE) endogenous thrombin potential (ETP) upon ellagic acid activation was 1,727 (170) nM min (Figure 1A) in the 1st week after intubation. Using linear mixed models, ETP in a crude model was significantly lower with −541 nM min (95%CI: −1,011 to −73) in the 2nd week, but in none of the other weeks after intubation compared to the first week (Table 3, model 1). After adjustment for age, sex, body mass index, APACHE-II score, cardiovascular disease, and smoking, ETP was significantly lower by −522 nM min (95%CI: −1,029 to −15) in the 2nd week, but in none of the other weeks after intubation compared to the first week (Table 3, model 2). Additional adjustment of model 2 by serial C-reactive protein, anti-Xa level, fibrinogen, SOFA score, pulmonary embolism and deep venous thrombosis, showed lower ETP in the 2nd [−724 nM min (95%CI: −1,143 to −304), the 3rd [−581 nM min (95%CI: −967 to −196)], and the 5th [−468 nM min (95%CI: −932 to −5)] weeks after intubation compared to the first week (Table 3, model 3). Overall, both statistical models (model 2, model 3) were not associated with significant changes in thrombin generation parameters over the course of the study.

The mean (SE) peak height (PH) upon by ellagic acid activation was 353 (45) nM (Figure 1B) in the 1st week after intubation. Using linear mixed models, peak height in a crude model showed no statistically significant difference over time since intubation. Adjustment for age, sex, body mass index, APACHE-II score, cardiovascular disease, and smoking status did not change this result (Table 3, models 1–2). Additional adjustment of model 2 by serial anti-Xa level, fibrinogen, C-reactive protein, SOFA score, pulmonary embolism and deep venous thrombosis, showed a reduced peak height in the 2nd [−158 nM (95%CI: −260 to −57)], and in the 3th [−123 nM (95%CI: −216 to −29)] week after intubation compared to the first week (Table 3, model 3).

Tissue factor triggered thrombin generation

The mean (SE) ETP upon tissue factor trigger was 1,620 (460) nM min (Figure 1C) in the 1st week after intubation. Using linear mixed models ETP in a crude model was not significantly different in weeks 2–6 since intubation compared to the first week. Adjustment for age, sex, body mass index, APACHE-II score, cardiovascular disease, and smoking did not change this result (Table 4, models 1–2). Additional adjustment of model 2 by serial C-reactive protein, anti-Xa level, fibrinogen, SOFA score, pulmonary embolism and deep venous thrombosis, showed a reduced ETP of −470 nM min (95%CI: −890 to −50) in the 3rd week, but in none of the other weeks, after intubation compared to the first week (Table 4, model 3).

The mean (SE) peak height for thrombin generation triggered by tissue factor was 264 (96) nM thrombin (Figure 1D) in the 1st week after intubation. Using linear mixed models the peak height in a crude model, was significantly higher in the 4th week after intubation compared to the reference value [116 nM thrombin (95%CI: 17–214); Table 4, model 1]. After adjustment for age, sex, body mass index, APACHE-II

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TABLE 2 Number of COVID-19 patients, the incidence of therapeutic anticoagulation, routine laboratory measurements, SOFA-score, and anti-Xa levels per week starting from intubation.

Parameters	Week number					
	1	2	3	4	5	6
Number of patients (n)	13	14	22	16	14	10
Percentage of patients treated with TA (%)	46	68	82	81	92	78
CRP (mg/L)	28 (9–134)	37 (24–115)	83 (41–159)	114 (45–199)	135 (45–231)	305 (220–360)
Fibrinogen (g/L)	5.7 (4.5–7.5)	5.4 (4.8–6.1)	6.1 (5.3–7.9)	7.7 (6.5–8.5)	8.2 (6–9)	8.9 (8.1–9)
SOFA-score	3 (2–5)	6 (4–7)	6 (3–7)	6 (3–8)	6.5 (4–9)	7 (6–8)
Anti-Xa (IU/ml)	0.68 (0.32)	0.57 (0.25)	0.53 (0.11)	0.53 (0.26)	0.58 (0.18)	0.51 (0.2)

TA, therapeutic anticoagulation; CRP, C-reactive protein; SOFA-score, Sepsis-related Organ Failure Assessment.

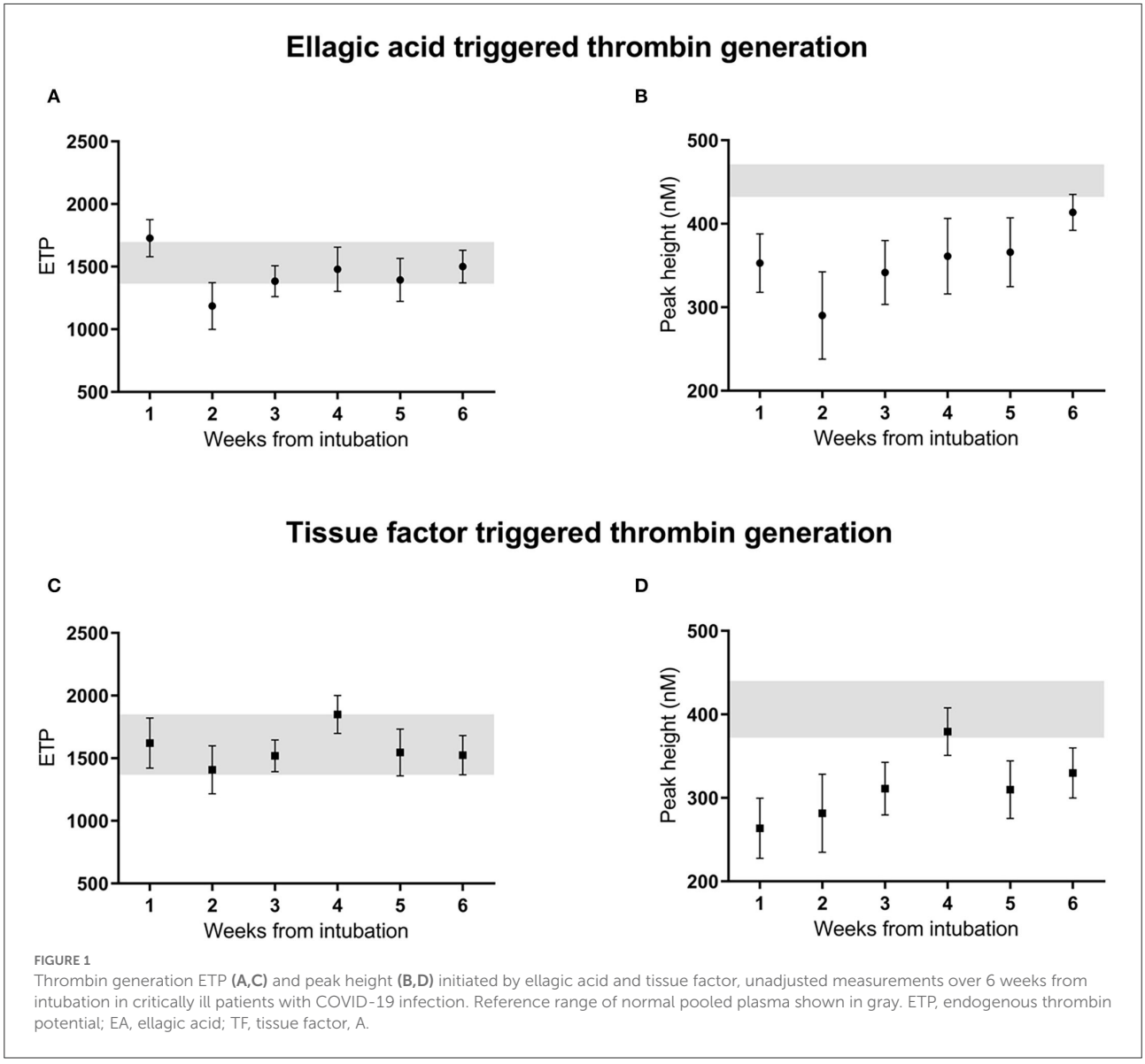

FIGURE 1
Thrombin generation ETP **(A,C)** and peak height **(B,D)** initiated by ellagic acid and tissue factor, unadjusted measurements over 6 weeks from intubation in critically ill patients with COVID-19 infection. Reference range of normal pooled plasma shown in gray. ETP, endogenous thrombin potential; EA, ellagic acid; TF, tissue factor, A.

TABLE 3 Ellagic acid-induced thrombin generation parameters, differences over time.

Model	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
ETP						
1	Ref	−541 (−1,011 to −71.6)*	−342 (−773 to 87.9)	−247 (−703 to 208)	−333 (−802 to 137)	−226 (−739 to 287)
2	Ref	−522 (−1,029 to −14.6)*	−179 (−632 to 275)	14.5 (−479 to 508)	−121 (−614 to 371)	191 (−416 to 799)
3	Ref	−724 (−1,143 to −304)*	−581 (−967 to −196)*	−415 (−840 to 11.2)	−468 (−932 to −5.00)*	−270 (−792 to 252)
Peak height						
1	Ref	−62.9 (−187 to 61.2)	−11.3 (−125 to 102)	8.23 (−112 to 129)	13.0 (−111 to 137)	60.6 (−74.7 to 196)
2	Ref	−99.2 (−233 to 34.6)	−8.47 (−128 to 111)	39.6 (−90.7 to 170)	24.5 (−105 to 154)	109 (−51.6 to 269)
3	Ref	−158 (−260 to −56.6)*	−123 (−216 to −29.1)*	−87.0 (−190 to 16.3)	−81.3 (−194 to 31.1)	−25.8 (−152 to 101)

Model 1 = unadjusted. Model 2 = adjusted for age, gender, body mass index, APACHE II, cardiovascular disease, smoking. Model 3 = adjusted according to model 2 + serial C-reactive protein, anti-Xa level, fibrinogen, SOFA score, pulmonary embolisms and deep venous thrombosis. * $P < 0.05$ compared to reference.

TABLE 4 Tissue factor-induced thrombin generation parameters, differences over time.

Model	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
ETP						
1	Ref	−212 (−707 to 283)	−102 (−542 to 338)	229 (−245 to 703)	−75 (−549 to 399)	−95 (−604 to 413)
2	Ref	116 (−431 to 663)	106 (−350 to 562)	384 (−105 to 873)	287 (−209 to 782)	500 (−101 to 1,101)
3	Ref	−311 (−790 to 168)	−470 (−890 to −50)*	−123 (−570 to 323)	−130 (−629 to 368)	3 (−551 to 557)
Peak height						
1	Ref	18.0 (−84.9 to 121)	47.6 (−43.8 to 139)	116 (17.2 to 214)*	46.3 (−52.2 to 145)	66.4 (−39.3 to 172)
2	Ref	56.2 (−60.4 to 173)	65.7 (−31.6 to 163)	125 (20.7 to 229)*	93.6 (−12.1 to 199)	138 (10.3 to 267)*
3	Ref	−34.1 (−133 to 64.6)	−65.3 (−152 to 21.3)	14.1 (−77.9 to 106)	−0.95 (−104 to 102)	24.4 (−90.0 to 139)

Model 1 = unadjusted. Model 2 = adjusted for age, gender, body mass index, APACHE II, cardiovascular disease, smoking. Model 3 = adjusted according to model 2 + serial C-reactive protein, anti-Xa level, fibrinogen, SOFA score, pulmonary embolisms and deep venous thrombosis. * $P < 0.05$ compared to reference.

score, cardiovascular disease, and smoking, the peak height was significantly higher in the 4th [125 nM (95%CI: 21–229)] and the 6th week [138 nM (95%CI: 11–267)] after intubation compared to the first week (Table 4, model 2). Additional adjustment of model 2 by serial C-reactive protein, anti-Xa level, fibrinogen, SOFA score, pulmonary embolism and deep venous thrombosis showed no statistically significant difference over the weeks since intubation (Table 4, model 3). Overall, both statistical models (model 2, model 3) were not associated with significant changes in thrombin generation parameters over the course of the study.

Patterns over time for the parameters lag time (start of the curve), velocity index (upward slope of curve) and time to tail (end of curve), were in agreement with results for ETP and peak height for both ellagic acid and tissue factor triggered thrombin generations (data not shown).

Additional attenuation of coagulation

The obtained thrombin generation data suggest a restricted anticoagulant potential of LMWH in plasma from severe COVID-19 patients. For both the ellagic acid and tissue factor triggered thrombin generation, the ETP for COVID-19 plasma was within the reference range of normal pooled plasma and the peak height was only slightly lower, despite being on therapeutic anticoagulants (Figure 1). Overall, in most assessed COVID-19 plasma aliquots, thrombin generation was only slightly affected by the presence of LMWH.

Comparing normal pooled plasma with COVID-19 pooled plasma (0.3 IU/ml LMWH) both (additionally) spiked with LMWH from 0.15 to 1.2 IU/ml showed an overall higher tissue factor triggered thrombin generation profile for the COVID-19 pooled plasma at all heparin levels (Figures 2A,B). Adding 0.3 IU/ml LWMH to normal pooled plasma reduced the peak height by ∼50%. Spiking this plasma with an additional 0.6 IU/ml LMWH reduced the peak height further from 180 to 55 nM thrombin. In comparison, adding 0.6 IU/ml LMWH to COVID-19 pooled plasma (containing 0.3 IU/ml LMWH) reduced the peak height by only ∼35% from 300 to 180 nM thrombin.

Additional inhibition of factor Xa with rivaroxaban on top of the 0.3 IU/ml LMWH showed comparable reduction profiles for both the normal and COVID-19 pooled plasma (Figures 2C,D); similarly, the anticoagulant potential of danaparoid was comparable for normal and COVID-19 pooled plasma (Figure 3).

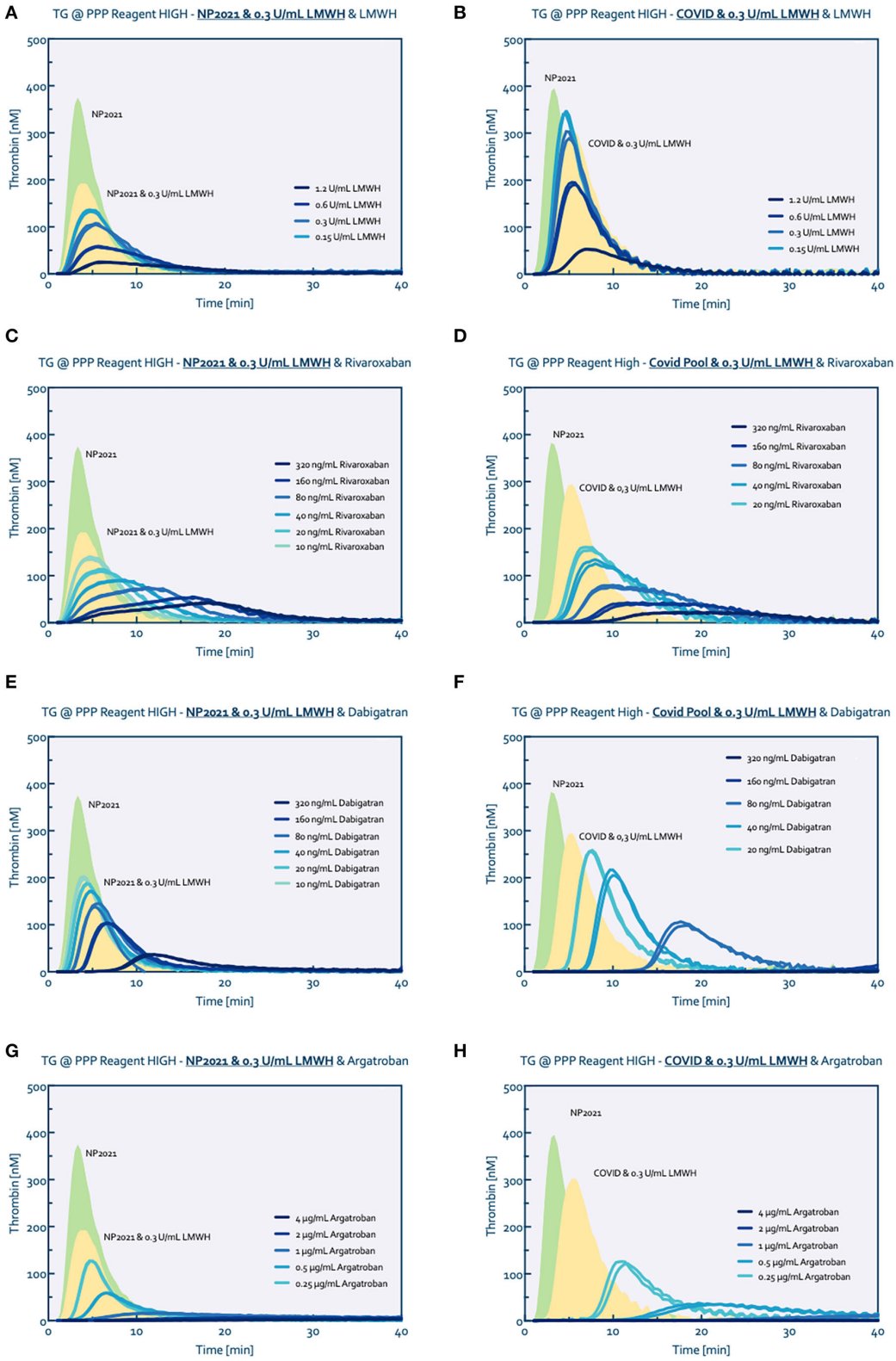

FIGURE 2
Thrombin generation titrated with several anticoagulants. Normal pool plasma (NP) was measured without (green curve) and with the addition of 0.3 U/mL LMWH (yellow curve) to match the baseline conditions of the COVID pool plasma (0.3 U/ml LMWH). Thrombin generation was consecutively measured after spiking with LWMH **(A,B)**, Rivaroxaban **(C,D)**, Dabigatran **(E,F)** and Argatroban **(G,H)**, respectively.

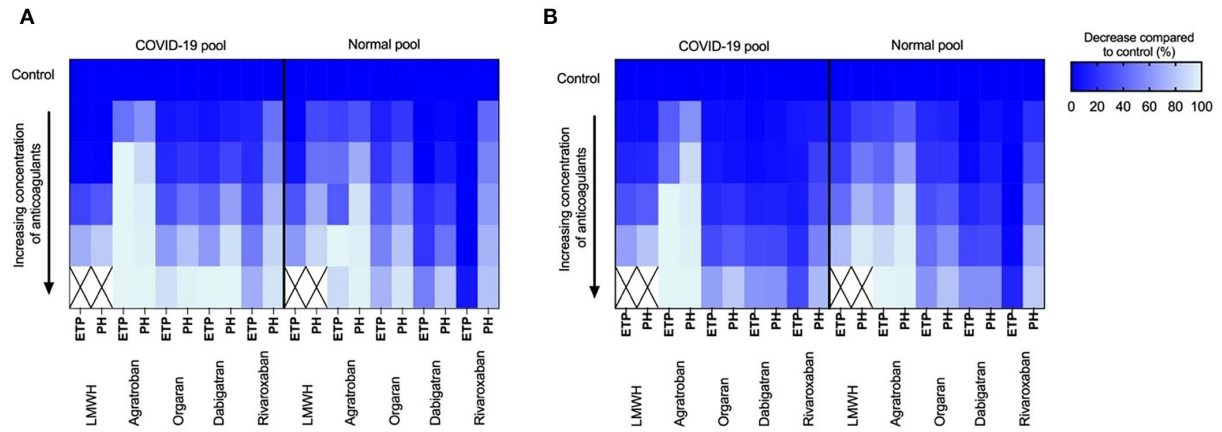

FIGURE 3
Tissue factor and ellagic acid triggered thrombin generation showing dose dependent inhibition of coagulation by low-molecular-weight-heparins (LMWH; 0.3–1.2 IU/ml), Argatroban (0–4 μg/ml), orgaran (0–1.6 U/ml), dabigatran (0–320 ng/ml) and rivaroxaban (0–320 ng/ml) in normal pool plasma and COVID-19 pool plasma spiked with 0.3 IU/ml LMWH. Color scheme indicates the reduction (0–100%) in endogenous thrombin potential (ETP) and peak height (PH) upon increasing concentration of inhibitors compared to control (100%). **(A)** Inhibition of tissue factor triggered thrombin generation and **(B)** Inhibition of ellagic acid triggered thrombin generation.

In contrast, thrombin inhibition by either dabigatran or argatroban in combined with 0.3 IU/ml LMWH showed a higher anticoagulant potential in COVID-19 pooled plasma than the normal pooled plasma (Figures 2E,F for dabigatran, Figures 2G,H for argatroban). The addition of 80 ng/ml dabigatran to COVID-19 pooled plasma prolonged the lag time from 3 to 14 mins and reduced the peak height from 290 nM to 100 nM thrombin. Comparable to dabigatran, 0.25 μg/ml argatroban prolonged the lag time to 8 mins and reduced the peak height to 125 nM thrombin. In comparison, 80 ng/ml dabigatran in normal pooled plasma prolonged the lag time by 1.5–3 mins and reduced the peak height by 50 nM from 190 to 140 nM thrombin. In the presence of 0.25 μg/ml argatroban, thrombin generation in normal pooled plasma (with 0.3 IU/mL LMWH) was only prolonged by 1 minute with a peak height reduced to 130 nM.

Comparable results were obtained for the ellagic acid triggered thrombin generation, with additional anticoagulant potential of thrombin inhibition in COVID-19 pooled plasma (Figure 3).

Discussion

The study has resulted in five main findings; First, anti-Xa levels remained within therapeutic range throughout the ICU stay despite the varying number of patients on therapeutic and prophylactic anticoagulation per week. Second, the coagulation potential measured by the thrombin generation assay by both ETP and peak height remained high over the course of six weeks after intubation, despite adequate anti-Xa levels. This observation is independent of sex, age, body mass index, APACHE-II, cardiovascular disease, and smoking status. Third, persistently high coagulation potential by thrombin generation was lower after additional adjustment for serial C-reactive protein, anti-Xa level, fibrinogen, SOFA score, incident pulmonary embolism and deep venous thrombosis. This implies, at least a partial role of inflammation, coagulation, and multi-organ failure in the observed association between time and ETP and peak height of thrombin generation in patients on heparin or LMWH. Fourth, the results of persisting high coagulation potential seem to be most evident in ellagic acid-initiated thrombin generation assays. This, in combination with the evidence of contact activation by neutrophil extracellular traps (NETs) in COVID-19 could imply an important role for the intrinsic coagulation cascade and contact activation system in COVID-19 associated coagulopathy. Finally, in vitro addition of thrombin inhibitors to COVID-19 pooled plasma showed a significantly increased anticoagulant potential compared to LMWH only, suggesting a benefit of direct thrombin inhibitors in reducing COVID-19 coagulopathy.

In the present study we have noticed a variable percentage (46–92%) of patients with an indication for therapeutic anticoagulation using LMWH or UFH over the weeks during ICU admission. The accompanying anti-Xa levels were within the therapeutic range suggesting that anticoagulation was effectively dosed. However, whether this dose is effective enough in reducing the incidence of thrombotic complications in COVID-19 patients remains unclear. In the MaastrICCht cohort the incidence of pulmonary thromboembolism is 33% (18), which was in line with other comparable cohorts (20, 41–43) of mechanically ventilated COVID-19 patients. However, our

data on thrombin generation suggests that this anticoagulant treatment does not adequately reduce thrombin generation in COVID-19 patients, which may contribute to clinical thrombosis. This is in line with the observations made in other studies (44, 45).

Our analyses revealed that elevated thrombin generation potential persisted over time independent of clinical characteristics and disease severity scores. The ongoing thrombophilic state could only be explained to a small extent by clinical markers of inflammation, coagulation, and multi-organ failure (46). Inflammation, disease severity and thromboembolic complications seem to explain thrombin generation peaks at least partially in the first three weeks of admission as displayed by model 3. In contrast, model 2 showed some changes in weeks 4 and 6 possibly suggesting an influence of patient characteristics in disease recovery.

In addition, the coagulation potential in COVID-19 patients remained high over the course of 6 weeks after admission to the ICU despite adequate anti-Xa levels. However, the coagulation potential of the COVID-19 population would most likely be even higher without LMWH. The inability to normalize the thrombin generation potential may reflect a mechanism leading to heparin resistance, driving the hypercoagulable state in CAC (23, 47). Interestingly, persisting increases in thrombin generation have been reported even up to a year after discharge, possibly caused by underlying mechanisms of persistent endothelial damage (48–51).

We measured thrombin generation initiated by either intrinsic (EA) or extrinsic (TF) activation of coagulation. Although a similar pattern of both ETP and peak height was seen throughout admission, some differences were observed. EA initiated thrombin generation showed significant changes in peak height throughout weeks 2 and 3 whereas the TF initiated thrombin generation did not. Thrombin generation was lower for both ETP and peak height in week 2, which then gradually increased again over time. This pattern over time was similar after adjustment for anti-Xa and C-reactive protein, suggesting an alternative mechanism that drives coagulation potential. Possibly NETs and histones are important as the contact activation pathway is activated by neutrophilic extracellular traps (NETs) and histones. This can lead to increased use of coagulation factors and inhibitors (9). In fact, our models adjusted for clinical markers of inflammation might be too non-specific to detect evidence for such an inflammatory mechanism. Another important observation were the flattened, prolonged thrombin generation curves in combination with a prolonged time to tail. These characteristics can be seen in samples with impaired antithrombin activity. It has been shown that histone citrullination and reduced levels of antithrombin play a significant role in COVID-19 disease compared to non-coronavirus sepsis patients in the ICU (52). However, also antithrombin can be citrullinated, decreasing its effective biological activity even further (53). Lowered and dysfunctional antithrombin could in part explain the insufficient inhibitory potential of heparin in severe COVID-19 patients as heparin inhibits thrombin and FXa by potentiating antithrombin binding. Our findings also support this, showing an insufficient decrease in thrombin generation potential during adequately dosed heparin treatment while we found a potential additional anticoagulant value of anticoagulants acting independently from antithrombin. The hypothesis of increased heparin resistance is further strengthened by recent observations of Benoit et al. who found no significant increases in thrombin generation after neutralization of heparin in COVID-19 patients (54).

Based on our *in-vivo* measurements of thrombin generation we evaluated several other possible anticoagulant treatments *in vitro*. The supplementation with additional anti-thrombin dependent anticoagulants (LMWH, danaparoid) was significantly less effective in COVID-19 patients compared to control plasma, supporting the hypothesis of lowered and dysfunctional antithrombin in these patients. Interestingly direct thrombin inhibitors (dabigatran, argatroban) were more effective anticoagulants in COVID-19 plasma compared to spiked normal pool plasma, possibly due to a high amount of fibrin-bound thrombin which remains biologically active while being shielded from some of the natural inhibitors but not anticoagulants. For this reason, we think that (direct) thrombin inhibitors may offer an attractive therapeutic tool as anticoagulant treatment of CAC and possibly other inflammation-mediated coagulopathies.

To our knowledge, we present one of the few studies describing serial assessment of thrombin generation follow-up for multiple weeks after ICU admission that have been reported in the current literature. Limitations to our study mainly involve its generalizability and applicability in clinical practice. Due to the build-up of our cohort, not all patients were followed from ICU admission. As a result of this, we collected extensive follow-up over a long period, but conclusions concerning individual disease courses cannot be drawn from our data. The inclusion of all mechanically ventilated COVID-19 patients, irrespective of ECMO and CRRT, makes for a heterogeneous population, suggesting careful interpretation of the results and limited generalizability to other ICU populations. Additionally, because of the relatively small number of patients, no conclusions can be drawn regarding the risk for adverse outcome (VTE or mortality), which would have added to the study value. Finally our population is limited to first wave patients, which might hamper translation to patients admitted to ICU in current clinical practice.

Conclusion

We showed that, in a cohort of mechanically ventilated, critically ill COVID-19 patients on anticoagulation, despite apparent adequate anti-Xa levels, thrombin generation potential

remains elevated. Elevated thrombin generation potential persists over the course of ICU admission and is independent of age, sex, body mass index, APACHE II score, cardiovascular disease, and smoking status. These observations may, at least in part, be explained by (anti)coagulation and thrombosis, inflammation, and multi-organ failure states. The lack of anticoagulant efficacy despite adequate anti-Xa levels suggests a mechanism of heparin resistance. Our *in vitro* assays indicate that antithrombin-dependent anticoagulants are relatively ineffective in reducing thrombin generation in COVID-19. In contrast, direct thrombin inhibitors show a promising anticoagulant potential compared to LMWH in severe COVID-19 patients, however more research is needed to confirm this. More in-depth investigation of mechanisms driving thrombin generation potential in COVID-19 is warranted to increase our understanding of CAC.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Medisch-Ethische Toetsingscommissie AzM/UM. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

TvB and MM analyzed and prepared the data and wrote the manuscript. TA performed statistical modeling and wrote a subsection regarding the statistical analysis. MN performed *in vitro* experiments and provided a visual representation of the results. RvO planned and oversaw all laboratory analyses within the study. EB and TH provided through feedback on the study and manuscript. A-MH provided feedback on the written manuscript. J-WS and IvH were involved in patient management and provided valuable feedback on the manuscript. HtC oversaw the study setup and provided valuable feedback on the manuscript. HS oversaw the experimental setup and was involved in manuscript writing. BvB was involved in patient management and oversaw the study and manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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External validation of the MidiCAT variant of thrombography: Comparison with calibrated automated thrombography and study of the centrifugation scheme

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Introduction: To perform Calibrated Automated Thrombography (CAT), the use of reduced plasma volumes (referred to as "MidiCAT") makes it possible to more efficiently use limited volumes of valuable biobanked plasma samples and decreases expenses for reagents. It is, however, unclear whether the MidiCAT procedure is suitable when thrombin generation (TG) is studied in the presence of added thrombomodulin (TG-TM). Moreover, a simplified centrifugation scheme would facilitate biobanking, if appropriate, for more sensitive coagulation studies. We aimed to compare the results of "MidiCAT" (halved plasma and reagent volumes) with those from regular CAT, in the absence or presence of TM, as well as to study the impact of a single-centrifugation scheme for plasma preparation before freezing.

Materials and methods: Plasma samples were prepared from the citrated blood from 20 Geneva hospital diverse patients without gross coagulation abnormalities with a single- or double-centrifugation scheme. Samples were kept frozen at −80°C and thawed just before the TG assay in duplicate under two conditions: 1 pM tissue factor (TF) or 5 pM TF + TM.

Results and discussion: (1) We externally validated "MidiCAT" and also extended the validation to TG-TM. Whatever the method (CAT or MidiCAT), intra-assay (assessed with duplicates) CV was below 6% (1 pM TF) or below 10% (5 pM TF + TM) for ETP. Agreement between the MidiCAT and CAT results was satisfactory; the p coefficients were above 0.95 for ETP and above 0.90 for most other parameters; biases for ETP were +10.0% (1 pM FT) and +13.5% (5 pM + TM). (2) The centrifugation scheme markedly affected the results obtained in the presence of TM, whereas the bias and limit of

agreement (difference plots) were low for the no TM condition. The bias in the presence of TM was obvious, more marked with plasma samples sensitive to TM when double centrifuged: the lower the ETP-TM, the greater the relative difference between the ETP-TM of plasma samples prepared with just single centrifugation and the reference plasma samples. Thus, a single-centrifugation procedure, as is often used for plasma biobanking, is suitable for TG study only if it is not performed in the presence of TM.

KEYWORDS

thrombin generation assay, blood coagulation tests, centrifugation, laboratory/methods, thrombomodulin

Introduction

Many researchers strive to obtain plasma samples that are suitable for clinical study of the biomarkers of the haemostatic system. Currently, the complicated regulatory administrative procedures, the ever-increasing financial costs, and the time required to include patients in prospective studies lead to delays in performing such studies. Biobanks are a crucial resource for medical research. They can give researchers access to large and varying ranges of laboratory data about a substantial number of patients. Samples in biobanks and data derived thereof could be used in cross-purpose studies involving separate research teams. However, for haemostasis tests, the use of such samples is not possible due to the specific and stringent pre-analysis requirements. Both standardization and simplification of pre-analysis procedures could facilitate biobanking, however, they should not come at the expense of the accuracy of the results.

To better predict thromboembolic events in patients with cancer, as well as prognosis and therapy response, haemostatic biomarkers are the subject of intense research. Many biobanks are specializing in the field of cancer. It thus seems very important to determine whether haemostasis tests can be performed with frozen plasma samples from cancer biobanks.

Many pre-analysis factors can influence coagulation assays, be they traditional or innovative ones, and for thrombin generation (TG) in plasma. The residual platelet count after centrifugation is regarded as one of the most important factors. The residual platelet count depends on both the applied centrifugal forces and the method used to harvest the plasma (1, 2). To eliminate as many platelets as possible, which may contribute to the variability in TG results, double centrifugation is the general rule (3–6). There have been attempts at standardization between laboratories (7), with much emphasis placed on the pre-analysis phase (8). However, some teams still work with single centrifugation, for example, those working in the fields of cirrhosis or multiple myeloma (9–12). It still seems uncertain whether a single-centrifugation scheme can be applied for tests such as the Calibrated Automated Thrombinography (CAT).

As regards tissue factor (TF) concentrations, 1 or 5 pM are frequently used. Regarding the assessment of the risk of thromboembolism, CAT performed in the presence of thrombomodulin (TM) is widely considered to be relevant (13, 14) since TM mobilizes the protein C system. This might be particularly relevant since poor sensitivity to activated protein C, often referred to as "acquired resistance" in contrast to V Leiden, has been widely considered an important mechanism in cancer-associated hypercoagulability. Indeed, it has now been well established that in complex acquired coagulation disorders, such as in cirrhosis or during the neonatal period, there are differences between both coagulation factors and natural anticoagulants, and normal adult plasma samples.

The limited availability of stored plasma is another limitation for laboratory investigations performed with biobanked plasma samples. Indeed, CAT testing needs more plasma than most other haemostasis tests (duplicates require 320 μL of plasma). The MidiCAT procedure could be a convenient way to analyse plasma samples with limited volumes of biobanked plasma (14).

To address these issues, we first compared MidiCAT with CAT using frozen (after two centrifugations) thawed plasma samples; second, we compared a single-centrifugation to a double-centrifugation scheme before freezing the plasma, examining TG with MidiCAT.

Materials and methods

Study design, setting, and patients

This study was conducted in a university tertiary hospital (in Geneva). In line with a previous study about CAT pre-analytic variables (4), we picked up at random 20 plasma samples collected during daily clinical practice with enough volume left, with a few exclusion criteria as follows: (1) greatly extended prothrombin time and activated partial thromboplastin time (aPTT), (2) being under 18 years of age, (3) known as using anticoagulants, or (4) the patient explicitly refusing to give

their general consent. This study was considered as falling outside of the scope of the Swiss legislation regulating research on human subjects so the need for local ethics committee approval was waived.

Collection and handling of samples

Blood samples were collected into vacuum tubes containing 0.109 mol/L trisodium citrate 1 vol:9 vol of blood, that is, the collection tubes generally used for coagulation tests at the hospital (Vacutainer, Becton Dickinson). Blood samples were centrifuged within an hour after arrival at the laboratory. The first centrifugation (2,500 × *g* for 10 min, the standard centrifugation scheme in the laboratory) was performed by haemostasis technicians as per daily practice procedures. They harvested 70% of the supernatant above the buffy coat while taking care not to disturb the buffy coat so that cell contamination would be limited. The quality-assurance policy of the laboratory includes regular checks, which consistently document platelet count $<10 \times 10^9$/L. Half of the plasma volume was aliquoted into capped plastic tubes (Fischer-Scientific type MBP3464). The second centrifugation of the remaining half was performed by research technicians (2,500 × *g* for 15 min) and the supernatant was aliquoted into the same tubes. Plasma samples were stored at −80°C until testing. Commercial liquid normal pooled plasma (NPP; Cryocheck®, Cryopep, Montpellier, France, batches A1255 and A1260; citrate levels equivalent to 109 mM according to the purchaser) was used as a control. The centrifuge (Universal 320, Hettich GmbH, Tuttingen, Germany) was operated at 20°C using a light brake (15). All laboratory tests were performed within a week of storage. After being thawed for 5 min at 37°C, samples were immediately analysed.

Laboratory assays

Thrombin generation (TG) was studied using Calibrated Automated Thrombography (CAT–Diagnostica Stago, Asnières-sur-Seine, France) with Stago reagents using Fluoroscan Ascent Fluorometer (version 5.0, Thermolab Systems, Helsinki, Finland). Runs were performed at 37°C after a 10 min pre-heating in the fluorometer of the round-bottom 96-well plate (Immulon 2HB) loaded with plasma samples and with procoagulant phospholipids and TF (see below). Fluo-Buffer together with FluCa was then automatically dispensed leading to recalcification of citrated plasma so that the reaction could start. Fluo-Buffer is a Hepes solution (pH 7.35) with calcium chloride, whereas Fluo-Substrate contains a thrombin-specific fluorogenic substrate (Z-GGR-AMC) solubilized in DMSO. To prepare FluCa, Fluo-Substrate was added to the warmed Fluo-Buffer shortly before the experiment. FluCa was freshly prepared for each run. Each TG run was calibrated according to the fluorescence curve obtained from a sample of the same plasma supplemented with a Thrombin Calibrator, with the specified amount of thrombin–α_2–macroglobulin complex, and FluCa. Inner-filter effect and substrate consumption were accounted for as well. Fluorescence was recorded every 15 s for 60 min. All samples were analysed in duplicate. Parameters of interest were derived from each TG curve (i.e., thrombogram) using the Thrombinoscope software, version 5.0 (Diagnostica Stago, Asnières-sur-Seine, France). All experiments were conducted with the same batches of reagents. TM (rabbit lung; BioMedica Diagnostics Inc., Stamford USA) was added to the TG mixture to assess the dynamic inhibitory protein C system. We used a TM concentration of 1.75 nM to induce a 50% decrease in ETP compared with values in the absence of TM with normal plasma.

TABLE 1 Median values and ranges of thrombin generation studies with the reference method and MidiCAT variant.

TG parameters	Plasma preparation	
	CAT 80 µL plasma	MidiCAT 40 µL plasma
1 pM TF		
ETP (nM × min)	1,359	1,160
	(417; 1,743)	(326; 1,626)
Peak (nM)	146	134
	(39; 209)	(27; 193)
Lag time (min)	6.7	5.7
	(4.7; 13.2)	(3.7; 12.7)
TTP (min)	11.8	10.3
	(9.8; 18.3)	(7.0; 17.3)
5 pM TF + TM		
ETP (nM × min)	1,035	879
	(0.0; 1,591)	(0.0; 1,452)
Peak (nM)	187	182
	(0.0; 314)	(0.0; 308)
Lag time (min)	4.7	3.9
	(2.7; 13.2)	(0.0; 9.5)
TTP (min)	7.5	6.7
	(5.0; 18.3)	(0.0; 15.7)

TG was initiated with 1 pM TF alone and with 5 pM TF combined with TM with 10 patients' plasma samples. CAT, calibrated automated thrombography; ETP, endogenous thrombin potential; TTP, time to peak; TF, tissue factor; TG, thrombin generation; TM, thrombomodulin.

First, we compared MidiCAT with the CAT method using frozen (after two centrifugations)–thawed plasma samples. Second, we compared the TG of plasma samples prepared with either a single-centrifugation scheme or a double-centrifugation scheme using MidiCAT.

For the CAT method acting as reference, experiments were performed at a total volume of 120 µL. To initiate TG, 20 µL of the reagent comprising procoagulant phospholipid

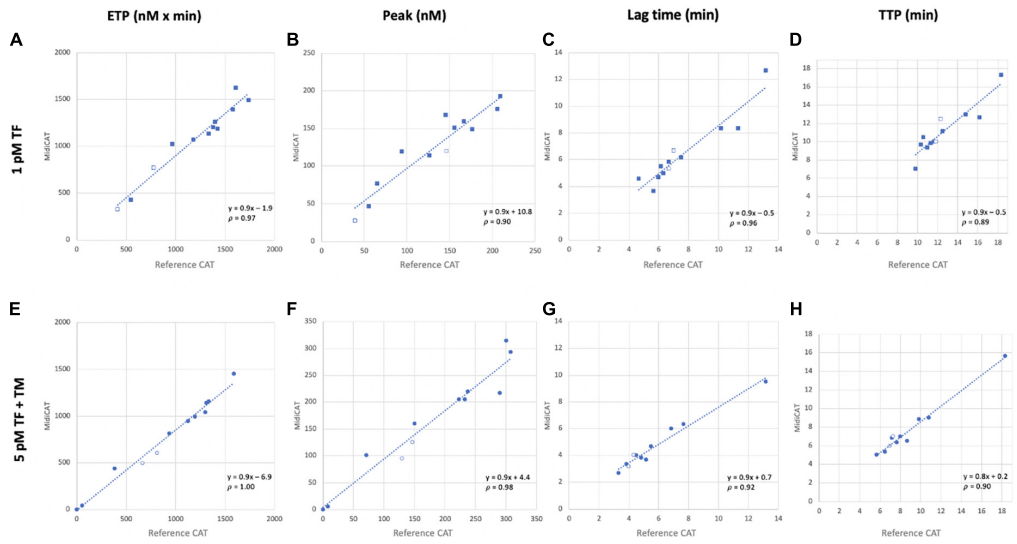

FIGURE 1
Correlation of thrombin generation parameters obtained with MidiCAT variant method with those of the reference method. Coagulation was initiated with 1 pM **(A–D)** and 5 pM TF in the presence of TM **(E–H)**. Plots for endogenous thrombin potential [ETP, nM × min; **(A, E)**], peak [nM; **(B, F)**], lagtime [min; **(C, G)**], and time to peak [TTP, min; **(D, H)**]. Filled dots: patients' plasma samples; empty dots: commercial normal pooled plasma. Plasma's volume used: 40 μL for MidiCAT method and 80 μL for CAT method. CAT, calibrated automated thrombography; TF, tissue factor; TM, thrombomodulin; p, Spearman's rank correlation coefficient.

vesicles only (MP-reagent) or recombinant TF and procoagulant phospholipid artificial vesicles (either PPP-low reagent 1 pM TF or PPP reagent 5 pM TF) were added to 80 μL of plasma into each well. TG was eventually triggered with automated dispensing of 20 μL FluCa reagent.

For the MidiCAT method (Bloemen et al. (16)), experiments were conducted in a reduced total volume of 60 μL. All volumes of the CAT method were halved. To modify the dispensing volume, we went to the folder: C: \Program Files (x86) \Thrombinoscope \Users \(username) \Setting, opened the file: default.set with notepad located the line "nDispenseVolume", and entered the desired volume.

Statistical analysis

Median values (interquartile ranges, IQR) and figures (percentages) were used for descriptive purposes. Correlations between the results of TG obtained with the two CAT methods and with the two centrifugation schemes were determined with Spearman's coefficient (p). To further evaluate the differences and biases in results between the two procedures, we plotted the percentage difference between the two methods ((B-A)/A × 100) against the ETP obtained with the reference method (methods A and B: reference and alternative procedure, respectively). Coefficients of variation (CV) were calculated for inter-well imprecision (standard deviation/mean × 100). The graphs and analyses were done using R software (version 3.5.0).

Results

To first compare MidiCAT and CAT and then compare the single-centrifugation and double-centrifugation schemes, plasma samples from 20 patients were analysed. In addition, two commercial normal plasma pools were studied both with CAT and MidiCAT. On the whole, 133 duplicate thrombograms were obtained under different conditions. One half of the patient samples was used for the first part of the study and the other for the second part.

The median age of patients was 46 years (range: 24–89). There were eight female patients. Prothrombin times were all within the normal range, and the median aPTT was 25.5 s (normal range: 23.6–32.5 s).

Regardless of the method used (CAT or MidiCAT), intra-assay (assessed with duplicates) CV was below 6% for ETP with 1 pM TF and below 10% with 5 pM TF in the presence of TM.

Comparison of the calibrated automated thrombography and MidiCAT methods

First, we compared MidiCAT with CAT using frozen (after two centrifugations)–thawed plasma samples under two experimental conditions: (1) low TF, to get the involvement of intrinsic tenase (anti-haemophilic factors) and (2) 5 pM TF in the presence of TM (at a concentration which halves the ETP

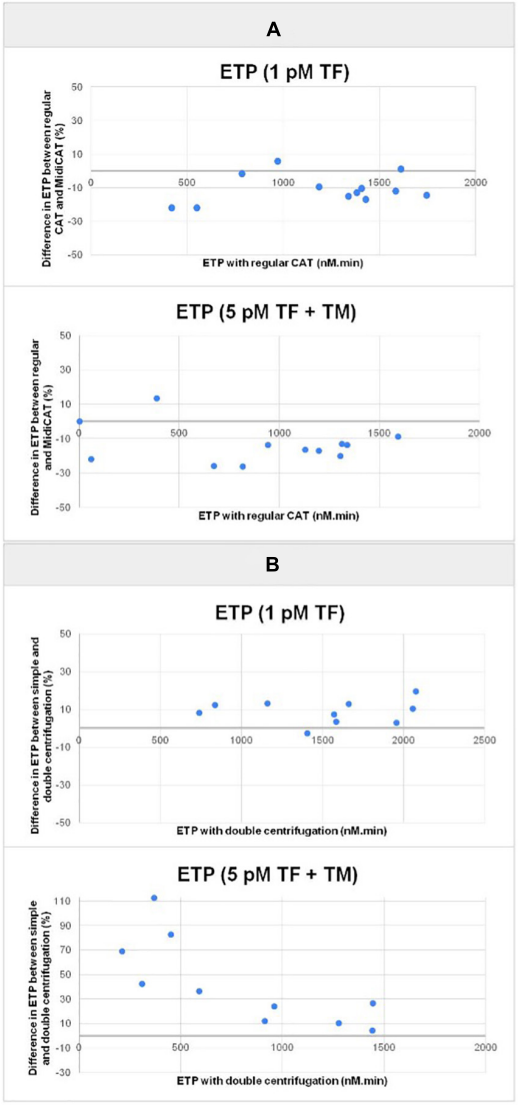

FIGURE 2
Plots of the difference in endogenous thrombin potential (ETP) between the variant methods and the reference method in percentage *vs.* endogenous thrombin potential (ETP) obtained with the reference method. TG was initiated with 1 pM TF alone, and with 5 pM TF combined with TM with 10 plasma samples (patients). Panel **(A)** comparison of MidiCAT variant method (40 μL plasma) with the reference method (CAT, 80 μL plasma)–plasma samples prepared with double centrifugation. Panel **(B)** comparison of single centrifugation and double centrifugation when preparing plasma; MidiCAT method. CAT, calibrated automated thrombography; ETP, endogenous thrombin potential; TM, thrombomodulin.

of normal plasma), to investigate potential hypercoagulability. Numerical results are shown in Table 1. The correlation coefficients were above 0.95 for ETP (Table 1, Figure 1) and above 0.90 for the other parameters except for TTP with 1 pM of TF (Table 1). The biases for ETP (MidiCAT *vs.* CAT) were +10.0% (1 pM FT) and +13.5% (5 pM + TM) (Figure 2). ETP-TM of some plasma samples was high (hypercoagulable), as expected within the patient population. However, the ETP-TM of the other plasma samples would be undetectable, which was unexpected. This points to hypersensitivity to TM, the underlying reasons for which remain unknown.

Comparison of the two centrifugation schemes

As a general rule, plasma preparation consists of two sequential centrifugations. We compared a single-centrifugation scheme with the double-centrifugation scheme as a reference using the MidiCAT protocol to study TG. Numerical results are shown in Table 2. Correlation coefficients were above 0.95 for ETP (Table 2, Figure 2). Correlation coefficients were above 0.90 for peak and TTP. Bias and limit of agreement of the difference plots were low for the no TM value but in its presence, there was an obvious bias, which is more pronounced when plasma samples were sensitive to TM: the more sensitive the plasma, the greater the relative difference of ETP-TM of plasma samples prepared with just single centrifugation to the reference plasma with double centrifugation (Figure 2). Coefficients of variation

TABLE 2 Median values and ranges of thrombin generation studies–single *vs.* double centrifugation scheme to prepare plasma samples.

TG parameters	Plasma preparation before freezing	
	Single centrifugation	Double centrifugation
1 pM TF		
ETP (nM × min)	1,664	1,578
	(798; 2,486)	(738; 2,075)
Peak (nM)	258	239
	(89; 423)	(69; 333)
TTP (min)	6.8	7.2
	(5.0; 10.7)	(5.3; 11.7)
Lag time (min)	3.8	3.7
	(2.8; 10.7)	(2.7; 7.7)
5 pM TF + TM		
ETP (nM × min)	922	751
	(354; 1,829)	(210; 1,443)
Peak (nM)	207	168
	(62; 386)	(34; 299)
TTP (min)	6.8	7.8
	(5.0; 16.0)	(5.7; 25.3)
Lag time (min)	5.2	4.2
	(3.3; 23.3)	(3.0; 14)

TG was initiated with 1 pM TF alone and with 5 pM TF combined with TM with 10 plasma samples (patients). ETP, endogenous thrombin potential; TTP, time to peak; CAT, calibrated automated thrombography; TF, tissue factor; TG, thrombin generation; TM, thrombomodulin.

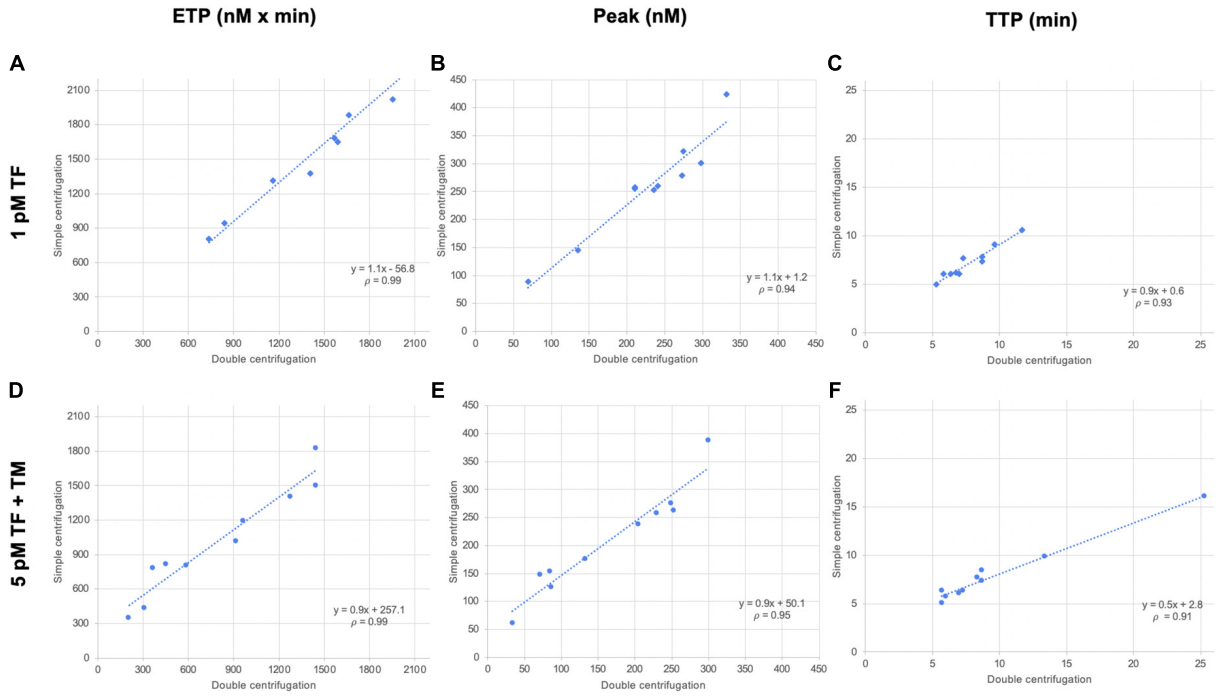

FIGURE 3
Correlation of thrombin generation parameters of plasma samples prepared with two centrifugation schemes. Ten plasma samples (from patients) were prepared with single centrifugation or double centrifugation, and TG was initiated with 1 pM TF and 5 pM TF in the presence of TM. Scattergrams of endogenous thrombin potential [ETP, nM × min; **(A, D)**], peak [nM; **(B, E)**], and time to peak [TTP, min; **(C, F)**]. TF, tissue factor; TM, thrombomodulin; p, Spearman's rank correlation coefficient.

were below 8% with regard to inter-well imprecision for ETP, peak, and TTP.

Additionally, contact phase activation in those plasma samples was estimated by studying TG without the addition of TF (MP-reagent: no TF; 4 μM phospholipids). When the results of this condition were compared with the ones where TF was added to get a final concentration of 5 pM, thrombograms were delayed and ETP was lower. The average differences in ETP were 39 and 77%, with single centrifugation or double centrifugation, respectively, indicating that the centrifugation scheme does indeed have a substantial effect on ETP. On average, ETP with no added TF was 60% greater in plasma samples prepared with a single centrifugation, than in those prepared with double centrifugation (**Figure 3**).

Discussion

Calibrated Automated Thrombography (CAT) is the monitoring of thrombin generation and decay in clotting plasma, in short TG. CAT is widely used in the research field of haemostasis. Although, some current issues, such as sample volumes, limit the use of CAT. Experts have recommended performing CAT at the very least in duplicate (6). A calibrator must be run in parallel with the sample in which TG is measured. This determines the required volume of plasma. We have reported in this paper an external validation of "MidiCAT" (halved plasma and reagent volumes), as well as demonstrating its use for TG-TM. The TG tests were performed mostly with 5 pM TF. However, to sensitize CAT to hypercoagulable states, experiments should be conducted with the addition of TM. It allows for the assessment of the effect of the PC system on TG, according to the pioneering work of Dargaud (8) and its use afterward (14, 17–20). Not adding TM in CAT assays might explain why several studies have failed to show a significant correlation with the risk of venous thrombosis. More and more teams choose the experimental condition with added TM to investigate hypercoagulability. Thus, the necessary plasma volume for each CAT measurement (with and without TM) exceeds 480 μL. Bloemen et al. demonstrated that low plasma volume (40 μL) can be used with 5 pM TF, provided that all other volumes which are to be added to the reaction wells are halved as well (16). However, their measurements were performed with in-house reagents and only with 5 pM TF. Our results are, to date and to the best of our knowledge, the only reported demonstration whose results obtained from thrombograms using low plasma volume, that is, "MidiCAT", do not deviate substantially from those obtained by CAT, the reference method. The results did indeed correlate well, when using the commercially available

reagents (Stago; 5 pM TF + phospholipids–PPP Reagent, with added TM; and 1 pM TF + phospholipids–PPP Low Reagent). Spearman's rank correlation coefficient for ETP is at least 0.97. Bloemen et al. achieved similar results with 5 pM and 0 pM procedures (16). The higher surface-to-volume ratio might explain, at least in part, the biases which were about +10% (MidiCAT *vs.* CAT). Moreover, it is important to note that costs are almost halved thanks to the decreased volumes of reactants.

There are many cancer biobanks, however, most of the time, the pre-analysis requirements before freezing for these biobanks do not correspond to pre-analysis requirements for haemostasis tests. We evaluated two centrifugation schemes to harvest plasma before freezing: the reference one, which consists of two sequential centrifugations (the goal is to leave as few platelets as possible, since they influence TG by providing procoagulant membranes and various internal proteins) (21); the simplified one, with just single centrifugation. Our data showed matching results between tested frozen plasma samples, after the two methods for all TG parameters obtained with MidiCAT when coagulation was initiated with 5 pM TF in the absence of TM, but not in the presence of the latter. These results are in line with those of Loeffen et al., who compared TG in plasma samples obtained from 12 healthy volunteers, centrifuged once at 2,000 × *g* for 15 min *vs.* 2,000 × *g* for 5 min followed by second centrifugation of harvested plasma at 10,000 × *g* for 10 min. No difference was reported when 5 pM TF was used to initiate TG (4). Loeffen et al., however, recommended a double-centrifugation scheme in presence of phospholipids to evaluate the level of contact activation on TG, probably because of their results with the low TF concentration (1 pM) or in the absence of added TF. Synthetic phospholipids contained in MP-reagent can be sufficient to enhance TG initiated by FXIIa in some patients (22). Surprisingly, in Loeffen's study, plasma collected in vacutainer tubes with no added TF showed no significant differences in TG results in contrast to our results. However, a significant increase in ETP was found in the single-centrifugation samples compared to the double-centrifugation ones with 1 pM TF (23). On average, when no TF was added, we observed an increase in ETP between single-centrifuged and double-centrifuged plasma. These differences may be explained by the fact that the participants in our study were patients and not healthy volunteers. Moreover, our second centrifugation was at 2,000 × *g* for 15 min and not 10,000 × *g* for 10 min. It should be noted that some patient plasma samples might undergo a TF exposure phenomenon due to residual platelets (4). However, this seems to have had no impact on TG results initiated with 5 pM TF. Thus, based on our results, we suggest the possibility of using centrifuged frozen samples, routinely and primarily collected for other purposes, to perform TG with the MidiCAT method in the presence of phospholipids with initiation at 5 pM TF. This possibility is worth considering in future studies. Contact phase activation is a major problem at lower picomolar TF concentrations and this effect can be more pronounced when only a single centrifugation is performed, as we have found while adding only procoagulant phospholipids without TF (MP-reagent from Stago) (data not shown).

Regarding the results in the presence of TM, they are consistent with those of Lisman's team (24) which showed that normally sensitive plasma may become resistant. What makes such plasma samples less sensitive to TM remains to be elucidated, but alterations to protein C and protein S are unlikely. Second centrifugation after plasma thawing has been proposed as an option, but it has not been investigated which undesirable component is removed in this way. It could be an option which was recently proposed (24). This remains to be independently confirmed.

We have to keep in mind that TG varies due to the conditions under which blood is collected, namely due to the tubes used and if the preparation/manipulation is carefully performed. Bias can be induced by contact activation, by residual platelets, and/or by impact quantification of the effects on analytical precision (4). Some researchers have estimated that not taking this into account may lead to an overestimation of TG (4). The use of CTI prevents contact phase activation. Although CTI has impracticalities and related costs, it can be omitted as long as TF is added to get a 5-pM concentration. We suggest that using frozen citrated plasma samples (without CTI) after single centrifugation is feasible. Of note, blood is often collected into vacuum tubes containing only citrate, as has been done in the current study. Results might differ when using other tubes, such as Monovette® Sarstedt (4). Unfortunately, such specific conditions (tubes and preparation) are difficult to comply with and preclude the use of existing frozen plasma samples collected after just one centrifugation. Conducting a large prospective study with CAT is also difficult due to such stringent pre-analysis constraints and the associated therein costs. In the present study, we focused on suitable pre-analysis conditions that could permit turning regularly collected biobanked samples into usable material for CAT studies. Being able to use such conditions would permit for broader CAT applicability in line with the availability of the newly commercialized analyser ST-Genesia, notably for centrifugation schemes (25).

Contrary to the previous study on centrifugation schemes (4), ours was conducted with patient plasma samples and not samples from healthy volunteers. In addition, the centrifugation scheme was the one used in daily practice. Thus, our results are relevant to daily practice and readily applicable to clinical research. Our study has several limitations though. First, the diseases that the patients suffered from were unknown, and so abnormal TG phenotypes could not be explained. Of note, there was a large variation in TM reduction among patients' plasma samples. Some plasma samples could have presented pre-activation for any reason or could have contained unnoticed anticoagulant drugs. Second, the double-centrifugation scheme

that was used as comparison (2,500 × *g* for 10 min) is the mandated one in our institutions. On the other hand, some teams have used a 15 min centrifugation and/or second centrifugation at 10,000 × *g*, thus we cannot exclude that this might have affected the results. Third, only MidiCAT was tested for the comparison of the centrifugation schemes. However, it is unlikely that different results have been obtained using these schemes with regular CAT. Eventually, TG measurement in presence of 5 pM TF without added TM was not included in our study. Other studies are needed to prove that the condition without the addition of TM can be used with plasma obtained after single centrifugation and with reduced volume, aka MidiCAT.

Whether the volume of plasma required for TG studies can be reduced is a significant issue of practical importance. We have provided the first external validation of the MidiCAT procedure and conclude that accurate measurement of TG curves is feasible with half of the volume of the reference CAT method. The second issue we addressed was the simplified but still acceptable pre-analysis conditions. With this study, we aim to raise awareness of the lack of impact of artifactual contact activation or residual platelets on TG when using reagents containing 5 pM TF. We believe that our results add sufficient data to the previous study to allow for the use of the MidiCAT method with 5 pM TF with TM or with 1 pM TF (now referred to as, respectively, intermediary and low picomolar concentrations of TF). Moreover, the use of single-centrifuged plasma samples from biobanks for MidiCAT with 5 pM TF seems acceptable, provided no TM is added. A double-centrifugation scheme is recommended otherwise.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Commission Cantonale d'Ethique de Genève (hospital general consent for blood from required standard analyses when clinical data are not required). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

EC, SC, and TL designed the study, performed the analysis, and wrote the manuscript. SC and EC performed the experiments and collected the data. SC, DG, PF, and BT participated in data analysis. All authors contributed to the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Thrombin generation assays to personalize treatment in bleeding and thrombotic diseases

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Treatment of bleeding and thrombotic disorders is highly standardized and based on evidence-based medicine guidelines. These evidence-based treatment schemes are well accepted but may lead to either insufficient treatment or over-dosing, because the individuals' hemostatic properties are not taken into account. This can potentially introduce bleeding or thrombotic complications in individual patients. With the incorporation of pharmacokinetic (PK) and pharmacodynamic (PK-PD) parameters, based on global assays such as thrombin generation assays (TGAs), a more personalized approach can be applied to treat either bleeding or thrombotic disorders. In this review, we will discuss the recent literature about the technical aspects of TGAs and the relation to diagnosis and management of bleeding and thrombotic disorders. In patients with bleeding disorders, such as hemophilia A or factor VII deficiency, TGAs can be used to identify patients with a more severe bleeding phenotype and also in the management with non-replacement therapy and/or bypassing therapy. These assays have also a role in patients with venous thrombo-embolism, but the usage of TGAs in patients with arterial thrombosis is less clear. However, there is a potential role for TGAs in the monitoring of (long-term) antithrombotic therapy, for example with the use of direct oral anticoagulants. Finally this review will discuss controversies, limitations and knowledge gaps in relation to the introduction of TGAs to personalize medicine in daily medical practice.

KEYWORDS

bleeding, personalized medicine, thrombin generation, thrombosis, hemophilia

Introduction

Hemostasis consists of a number of highly balanced processes to ensure blood flow and prevent unnecessary thrombosis and bleeding. A shift in this balance can lead to either of these conditions with associated morbidity and mortality, and an impairment in quality of life (1). In patients with thrombosis this balance has shifted to a state with increased activation of prothrombogenic factors while in patients with bleeding disorders it is associated with an inability to ensure sufficient stable platelet plug formation. Treatment of both disorders is highly standardized and is shaped according to evidence based medicine guidelines.

The most well-known coagulation related bleeding disorder, hemophilia A (HA), is associated with a high bleeding risk due to a deficiency of coagulation factor (F) VIII (2). Severe HA patients, who have a FVIII activity level of <1 IU/dL, are treated with prophylactic coagulation factor replacement therapy to prevent bleeding and subsequent joint damage (3). The schemes for prophylactic therapy are standardized and adjusted according to FVIII activity trough levels (4). However, some patients with adequate FVIII activity levels still experience bleeding symptoms (5). On the other hand, patients with thrombosis are treated with anticoagulant therapy, for example direct oral anticoagulants (DOACs) in venous thrombo-embolism (VTE) (6). The dosage of this therapy is based on large scale randomized controlled trials (RTCs) and it is effective to prevent recurrent thrombosis in most patients, without the introduction of bleeding complications. Nonetheless, some patients experience recurrent thrombosis despite adequate therapy compliance, while others experience life-threatening bleeding with the same therapeutic dosage (6). Therefore, despite current state-of-the-art evidence-based medicine, diagnosis and treatment of patients with hemostatic disorders is possibly suboptimal due to either insufficient treatment in one patient, while over-dosing in the other. Both introduces a risk for bleeding and thrombotic complications in the individual patient.

The assays to analyze patients with thrombotic and bleeding disorders consists of screening assays like the prothrombin time (PT) and activated partial thromboplastic time (APTT) and on confirmation assays of specific coagulation factors, like FVIII and protein C activity level determinations. Both kind of assays investigate a certain part of the coagulation cascade and do not take the intertwining processes into account. A global hemostasis assay can measure these multiple processes (7). Several global hemostasis assays exist (8), all with the idea to provide a more detailed impression of the individual patients hemostatic balance. The physician can use these parameters together with personal characteristics of the patient, like concomitant use of medication and comorbidities that interfere with coagulation, to provide a clinical applicable picture to eventually adapt therapy upon (9).

In this review we will discuss the recent literature about thrombin generation assays in relation to the management of bleeding and thrombotic disorders. Assays using whole blood or investigating fibrinolysis are beyond the scope of this review. For bleeding disorders, the main focus will lay on HA, as this is the most prevalent coagulation related bleeding disorder. For thrombotic disorders, the focus will lie on venous thrombo-embolic disorders and the treatment with DOACs, heparinoids and vitamin K-antagonists (VKA). Furthermore, we will discuss controversies, limitations and knowledge gaps in relation to the introduction of plasma-based global assays to personalize medicine in daily medical practice.

Thrombin generation assays

The first reports of manual thrombin generation assays (TGAs) were published in 1953 (10, 11). Generation of thrombin is the result of effective activation of procoagulant factors of coagulation. Thrombin is a pivotal enzyme in hemostasis, as its generation represents a rate limiting step in fibrin formation, amongst its other key functions in hemostatic processes (12). It also functions as initiator of anticoagulant processes through potentiation of protein C upon binding of thrombin to thrombomodulin. *In vitro* measurement of thrombin generation uses substrates specific for thrombin cleaving activity that release a chromogenic or fluorogenic signal, to represent the net balance between these processes. Upon activation of the coagulation pathways through addition of tissue factor, phospholipids and calcium, the production of thrombin is initiated and accelerates exponentially, slows down, until it reaches a plateau phase (13). This can be measured in time, which gives insight in the net result of the hemostatic capacity.

The process of thrombin generation is visually represented by the first derivative of the thrombin generation signal as the signal accumulates in time, when measured with a chromogenic or fluorescent substrate (**Figure 1**). Important parameters describing this curve include lag time (1), which describes the time between application of the trigger and the initiation of thrombin generation, time to thrombin peak (2) when the generation of thrombin has reached its maximum, thrombin peak height (3) evaluating maximum generated quantity of thrombin, area under the curve (AUC, also called endogenous thrombin potential (ETP), (4) where the total amount of thrombin generation is evaluated, as well as the velocity index (5) which describes the slope of the curve during the amplification phase. These parameters vary in close conjunction; an increased thrombin peak height is mostly accompanied by an increased velocity index and ETP, while time to thrombin peak and also lag time are often shortened.

The lack of standardization of test parameters and composition of reagents has hampered comparability and harmonization of thrombin generation results to a great extent.

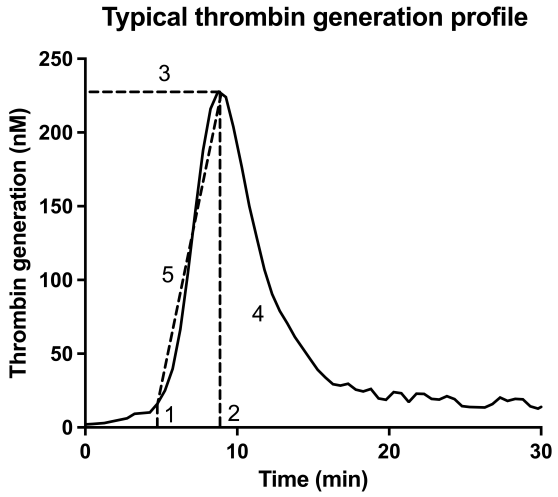

FIGURE 1
Essential parameters obtained with the thrombin generation assay. This thrombin generation assay is visually represented by the first derivative of the thrombin generation signal. The important parameters describing this curve include lag time (1), which describes the time between application of the trigger and the initiation of thrombin generation; time to thrombin peak (2) when the generation of thrombin is maximal; thrombin peak height (3) evaluating maximum generated quantity of thrombin; the area under the curve (AUC, also called endogenous thrombin potential (ETP), (4) where the total amount of thrombin generation is evaluated; as well as the velocity index (5) which describes the slope of the curve during the amplification phase.

First, the type of thrombin generation matrix varies from platelet poor plasma (PPP), platelet rich plasma (PRP) to whole blood. In most cases, PPP is used. For the TGA, standardization of preanalytical procedures to prepare PPP is essential. Comparison of different pre-analytical protocols where different blood collection systems, blood collection tubes, centrifugation method and time between collection and testing were evaluated, showed significant effects on thrombin generation results (14). Exemplifying centrifugation; a two-step centrifugation significantly decreased ETP potential compared to a one-step centrifugation. This is probably due to phospholipid contamination through microparticles or vesicles in samples that are centrifuged only once at a low centrifugation speed. Another factor that introduces pre-analytical variation is the venipuncture system and type of collection tube used. Addition of corn trypsin inhibitor (CTI) interferes with contact-activation, and thereby may reduce thrombin potential if contact activation occurs. However, this is also dependent on other factors like tissue factor concentration. The time and temperature at which a sample is preserved is another factor that can have a significant effect on thrombin generation results, as instability of coagulation factors does not allow prolonged storage times at room temperature. In conclusion, the establishment of a standardized pre-analytical protocol will aid significantly in harmonizing TGAs. The International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Subcommittee (SSC) suggested specific preanalytical conditions for the measurement of thrombin generation for the indication of hemophilia, which are described in ref (15).

Composition of the method that is applied to produce a fluorescent signal, is another important factor that hampers harmonization of thrombin generation results. Two main specific thrombin substrates have been applied to measure its generation, either attached to a chromogenic or fluorogenic signal, para-nitroaniline (pNA) and 7-amino-4-methylcoumar (AMC), respectively (16, 17). In both methods, the caged signal molecule is released by thrombin mediated proteolysis. The peptide is specific for thrombin and has a relative high Km and low Vmax to avoid substrate depletion. The chromogenic pNA signal is quantified through extinction measurement at 405 nM. Defibrination or inhibition of fibrin polymerization is required for this assay due to interference of the chromogenic signal during clot formation. Fluorescent AMC signal is not sensitive toward interference through clot formation and is excitated at a wavelength of 390 nm resulting in a peak at an emission wavelength of 460 nm. The rate at which these signal molecules are released varies between chromogenic and fluorescent substrates, and also the amino acid composition of the tri-peptide, which hampers comparison of results for these two methods (8).

Calibration of the assay can be performed by measurement of a known range of thrombin concentrations, or thrombin bound to alpha-2-macroglobulin (α2M). Fluorescent substrates in the TGA are also cleaved by thrombin complexed to α2M, whereas thrombin complexed to α2M is not biologically active in fibrinogen activation (18). Moreover, in specific patients, such as the pediatric population and neonates, the α2M concentrations can be significantly altered compared to the adult population (19). The biological active free thrombin component can therefore differ from the measured total thrombin activity. In some assays, the thrombin generation curve is corrected for the amount of thrombin that is bound to α2M, while in other assays no correction for α2M is applied/necessary (20).

Furthermore, correction for quenching of the fluorescent signal differs between methods. Correcting for quenching, or the inner filter effect, of the fluorescent signal in patient samples with varying composition is a non-linear phenomenon and may lead to an underestimation of thrombin generation and increased variation in test results (21). Some methods correct for quenching by the application of mathematical correction using an intra-assay control sample, in other methods no correction for quenching is applied (22).

Thrombin generation is initiated by the addition of tissue factor and phospholipids, which will activate the extrinsic pathway and stimulate coagulation, respectively. The concentration of these trigger components can differ and

will alter sensitivity of the assay toward different types of coagulation disorders. Reagent compositions applying low concentration of tissue factor and phospholipids are most sensitive toward bleeding disorders (23), whereas a higher tissue factor concentration can be predictive for a prothrombotic phenotype (24). In the anticoagulated patient, higher concentrations of tissue factor and phospholipids are suggested to be more appropriate (25). Tissue factor can be derived from either tissue (human plasma, human placenta, and rabbit), or through recombinant expression. Apart from the concentrations in trigger reagent, also the composition of the applied phospholipids (e.g., percentage of phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine) and the size distribution of the vesicles containing these phospholipids may vary. Imprecision in thrombin generation results is increased using different sources of tissue factor and phospholipids, especially at low concentrations (26). For most commercial available reagents, exact composition for the different reagents is not disclosed, and variation between batches could be expected (25).

Normalization of the obtained thrombin generation results, as suggested by the ISTH-SSC, is often advocated to reduce inter-assay and inter-laboratory variation, and to aid in the interpretation of the results of the different thrombin generation parameters (15, 26). However, determination and application of a normal sample is complex because no reference material exists, different pool samples, or commercially available control samples have varying composition and therefore may increase variation between assays as well as hamper comparability and interpretation of results of different assays.

Finally, thrombin generation can be determined with a manual, semi-automated or automated assay. The automated assays that are currently available for the evaluation of thrombin generation apply fluorescent substrates, but reagent composition, and calibration and mathematical procedures differ (8). The (semi-)automated assays that are often applied are the Calibrated Automated Thrombography (CAT, Stago), ST Genesia (Stago), and Ceveron TGA (Technoclone). A comparison of thrombin generation performance of these assays for patient populations with specific bleeding or thrombotic phenotypes, as well as patients treated with anticoagulants, has not been reported. Therefore, the effect of differences in reagent composition, calibration, correction methods and normalization on thrombin generation results cannot be interpreted for these patient population when different assays are used.

Bleeding disorders

TGAs are used in the analysis of bleeding disorders, to give an impression of the clinical phenotype of patients with bleeding disorders and to monitor treatment (for summary of most important findings, see **Table 1**). These different applications will be discussed here.

Diagnosis of bleeding disorders

Patients with a mild to moderate bleeding tendency are subjected to multiple diagnostic assays to detect the hemostatic abnormality (27). However, in one in three patients a definitive diagnosis cannot be established (28), a condition called "bleeding of unknown cause" (BUC) (29). Several research groups have investigated the additional value of thrombin generation measured in PPP in patients with BUC (30–36). These studies provided conflicting results, with two, older studies showing no association (30, 32), while other studies found abnormalities in thrombin generation parameters (31, 33–36). The studies that found decreased thrombin generation were generally larger than the studies that did not, and used lower TF concentrations to start the TGA. As a hypocoagulable state can best be detected with a low TF concentration, it is possible that the amount of TF can explain this difference. The most prevalent described abnormalities were a prolonged lag time (31, 33–36), decreased thrombin peak height (31, 33), and decreased ETP (31, 33, 34, 36). However, the main limitation of TGAs in BUC patients is the overlap in thrombin generation results between patients with BUC and healthy controls. Therefore, reference ranges for diagnosis of bleeding based on decreased thrombin generation or increased lag time need to be determined. A specific disease entity (for example, bleeding based on impaired thrombin generation, as data from previous mentioned studies imply) could improve scientific research and treatment for these patients.

Difference in clinical phenotype of bleeding disorders

The value of TGAs is best researched in patients with HA, where it shows an association with clinical bleeding phenotype. Only a few papers report about TGA and bleeding tendency in patients with rare bleeding disorders (RBDs) (37–39).

Hemophilia patients with the same genotypic variant and factor coagulation activity level often show differences in clinical bleeding phenotype, possibly due to alterations in other coagulation factors than FVIII (in HA) and FIX (in hemophilia B; HB). This hypothesis was confirmed by multiple small studies in which the bleeding phenotype correlated with one or multiple TGA parameters in PPP (40–44). One study was unable to establish this association (45), possible due to the use of the Tosetto bleeding score, that is developed for Von Willebrand disease (VWD), instead of a hemophilia specific bleeding score (46). Another study investigated the relationship between FVIII activity level, genetic variations and inhibitor status. Patients

with an inhibitor, a neutralizing antibody against FVIII making replacement therapy ineffective, showed decreased thrombin generation capacity compared to patients without inhibitors, despite equal FVIII activity levels (47). Thereby it can be concluded that TGAs can play a role in unraveling the clinical bleeding phenotype of hemophilia patients and even may play a role in how to treat these patients.

It is known that in patients with FXI deficiency (also known as hemophilia C) FXI activity level and bleeding phenotype do not correlate with each other (48). However, thrombin generation could possibly distinguish patients with different FXI activity levels and patients with and without bleeding. It appeared that certain sampling and testing conditions influenced the results of thrombin generation. In PPP with CTI, thrombin generation did not identify FXI deficient patients from normal controls. But when PRP with CTI and low TF was used, it could differentiate between patients with and without FXI deficiency. Furthermore, differences in thrombin peak height and thrombin potential had a high diagnostic accuracy for identifying bleeding from non-bleeding patients (49).

RBDs are a heterogenous group of diseases with different coagulation factor defects. All these diseases have a variable bleeding tendency, that only partially can be explained by the activity level of the missing or depleted coagulation factor. A retrospective study of RBD patients showed that thrombin peak height and ETP measured in PPP were significant lower in patients with major bleeding, compared to patients with minor bleeding (38). Major bleeding patients had ETP values <20% of normal (38). This was comparable to the results of another study that found that all patients with major bleeding had an ETP of <20% of normal, while RBD patients with an ETP >30% of normal had no clinically relevant bleeding symptoms (37). The added value of TGAs in patients with RBDs was confirmed in a third study which showed that it could better predict significant bleeding compared to factor activity level (39).

Lastly, a study involving patients with von Willebrand disease (VWD) showed that patients with a mild bleeding phenotype had higher thrombin peak height compared to patients with more severe bleeding. The thrombin peak height and velocity of thrombin generation both correlated with VWF activity level and FVIII activity levels. This was observed in both PRP as well as PPP. Plasma FVIII activity level was the main driver of thrombin generation in this study (50).

Treatment of bleeding disorders

Factor concentrate

The use of TGAs is mainly investigated in the treatment of HA patients. Spiking studies were first reported, in which plasma of HA patients was spiked with multiple concentrations of FVIII and thrombin generation in PPP was measured. Multiple *in vitro* spiking studies showed that thrombin generation reaches a plateau phase when FVIII activity level is between 20 and 50 IU/dL (40, 51–53), with only one study failed to show this plateau phase and suggested a linear correlation between FVIII activity level and thrombin generation (54). All studies reported major variation in the FVIII activity level at which thrombin generation was normalized in individual patients, indicating a need to individualize FVIII replacement therapy dosage to obtain normal thrombin generation in the TGA.

The individual response to FVIII replacement therapy was also reported in multiple *in vivo* studies with HA patients. A strong correlation between FVIII activity level and thrombin generation parameters (except lag time) in PPP was found (55–61), but the inter-patient variation of thrombin generation was large after a standard infusion of FVIII. For example, some patients generate normal total amounts of thrombin with subtherapeutic FVIII activity levels while others don't show normalization of thrombin generation despite adequate FVIII activity levels (56). On the other hand, the intra-patient variation was small, thereby suggesting that the thrombin generation in an individual patients is predictable (57, 60).

Furthermore, multiple studies showed that the thrombin generation response had a prolonged duration after a single bolus of factor VIII concentrate compared to FVIII activity level. FVIII activity level declined over time after administration of FVIII replacement therapy, while thrombin generation remained elevated (55, 57, 60, 62). This effect was, again, variable between patients, implicating that other factors, like very low-titer inhibitors or level of other coagulation factors could play a role. This was further investigated in a pharmacokinetic (PK)/pharmacodynamic (PD) modeling study which showed that on average a 50% ETP level (EC_{50}) was reached with only 11.6 IU/dL FVIII activity level increase. However, the inter-individual differences were large, underscoring the existence of an individual unique thrombin generation profile. For example, three patients with similar PK-profiles exhibited EC_{50} values that varied from 7.9 to 29.8 IU/dL FVIII (63).

A second PK/PD-modeling study also incorporated bleeding in their analysis. This study was based on the data of the GENA-21 study, which already had shown that FVIII activity level did not correlate with bleeding symptoms during prophylactic FVIII replacement therapy (64). However, patients with bleeding had significant lower thrombin generation compared to patients who had no bleeding symptoms (65). In this PK/PD-modeling study the authors found that patients with the highest ETP at baseline, had the lowest bleeding rate even with the lowest FVIII replacement therapy dosage compared to patients with the lowest ETP at baseline and highest FVIII replacement therapy dosage (66). This study showed that individualized dosing of FVIII replacement therapy based on ETP is superior in bleeding outcomes with this specific FVIII product.

In patients with hemophilia B (HB) a PK-PD model study was performed with a recombinant FIX-Fc fusion protein

(eftrenonacog-alfa) and showed that velocity of thrombin generation showed the best correlation with FIX activity level. Thrombin peak height and ETP were the following parameters that decreased over time after replacement therapy. However, bleeding was not assessed in this study (67).

Lastly, a cross-over study assessed the PK-PD relationship between supplementation of plasma derived (pd) FVII and recombinant activated FVII (rFVIIa) in patients with FVII deficiency. This study identified lag time as the best effect-response parameter. In the PD-analysis, it was shown that the EC_{50} was only 2 IU/dL FVII activity for both pdFVII and rFVIIa. Furthermore, they showed that a plasma FVII activity level of 3-4 IU/dL was sufficient to reach lag time values comparable with the upper limit of healthy controls (68). These data underscore the discriminating value of thrombin generation in RBDs, especially if supplementation therapy is difficult to monitor because of long turn-around times for certain coagulation factor activity level determinations or because of replacement with rFVIIa, activated prothrombin complex concentrate (aPCC) or plasma (see section "Bypassing agents").

Bypassing agents

Thrombin generation assays have an additional value in the monitoring of bypassing agents (BPAs), like rFVIIa and aPCC, in hemophilia patients. These products are mainly used in patients with inhibitors because FVIII replacement therapy is ineffective. Since BPAs cannot be monitored with a single factor assay, especially if it is given in combination with other treatment modalities, performing thrombin generation is an attractive alternative (9).

Activated prothrombin complex concentrates are shown to restore thrombin generation by spiking plasma samples of HA patients with 1–2 IU/mL aPCC (which corresponds with the therapeutic dose of 50–100 IU/kg) (69–71). In a PK/PD-study with aPCC in three HA patients, thrombin generation was restored after administration of 65–100 IU/kg aPCC and it diminished to 50% between 4 and 7 h (71). In pediatric HA patients with inhibitors, thrombin generation was restored to 80% of normal at peak aPCC levels after administration of 60–100 IU/kg. Thrombin potential remained enhanced 2.6 fold at trough aPCC level compared to control inhibitor plasma, indicating longer lasting effects on thrombin generation (72).

The effect of rFVIIa on thrombin generation was found to be less than aPCC in a cross-over study (73). The thrombin generation response after a bolus rFVIIa was highest after 30–60 min and decreased over a period of 4 h, anticipating the half-life of rFVIIa (74). All studies investigating the effect of rFVIIa in HA patients have shown that thrombin peak height and ETP are increased in PPP, but do not reach normal values (75, 76). Furthermore, individual patients show a difference in thrombin generation response to rFVIIa, with some patients having a poor response (76).

Since it is impossible to predict the hemostatic response of an individual patient to BPA therapy, Dargaud et al. proposed a three step model to individually tailor therapy. They investigated the performance of this model in six HA patients during ten invasive procedures. No bleeding occurred in the patients in whom ETP was normalized with the selected therapy (77).

Non-factor replacement therapy

During recent years, non-factor replacement therapies have been introduced in the treatment landscape of HA. Emicizumab, a bispecific monoclonal antibody that forms a pseudo-tenase complex (78), is the first non-factor replacement drug receiving market authorization, with other treatments like anti-tissue factor pathway inhibitor (anti-TFPI, e.g., concizumab) (79) and a mRNA against antithrombin (fitusiran) (80, 81) following pursuit. Because the hemostatic effects of these non-factor replacement therapies cannot be monitored with conventional assays, or lead to falsely normalized FVIII activity level (82), it is better to assess the end-product of hemostasis with TGAs, especially if used in combination with other treatment modalities (83).

The first study of emicizumab in FVIII depleted plasma showed thrombin generation parameters increasing to half of normal (82). One study found a linear correlation between emicizumab concentration and thrombin peak height measured in PPP (84). Moreover, results showed that thrombin potential in emicizumab treated HA patients reached a plateau at 20–30% of normal (84), which was replicated in other studies (85, 86). Moreover, it was shown that thrombin generation was lower in infants younger than 1 year, compared to older children and adults, possibly due to a faster clearance of emicizumab (85, 87, 88). Finally, thrombin peak height and ETP were significantly lower in patients who presented with major bleedings (85, 89).

TGAs can mainly be used to monitor combined treatment modalities, in which measurement of individual components of therapy is impossible. Because emicizumab was first investigated in HA patients with inhibitors, most data exist about the combination of emicizumab with BPAs. However, one *in vitro* spiking study showed that combination therapy of emicizumab with plasma derived FVIII/VWF (pdFVIII/VWF) did not increase thrombin generation above levels observed in PPP with monotherapy pdFVIII/VWF. This is expected because FVIIIa has a greater affinity for the tenase complex than emicizumab (90). Moreover, in multiple spiking studies (90–92), it was observed that combination therapy of emicizumab with aPCC in low dosage (5 IU/kg) already normalized thrombin generation. APCC in higher dosage (>30 IU/kg) increase thrombin generation above normal values, to even more than eight-fold normal values with a dosage of 100 IU/kg (90–92). On the other hand, when HA plasma with emicizumab was spiked with rFVIIa in the highest dosage of 270 μg/kg, thrombin generation did not exceed normal values (90–92). Most importantly, it was discovered that activated FIX, in aPCC

was responsible for the synergistic effect of emicizumab and aPCC *in vitro* (93). Whether this also occurs *in vivo* is still in debate. It can be concluded, however, that HA patients treated with emicizumab who need additional therapies should be treated with care and thrombin generation should be closely monitored. Patients without inhibitors can safely be treated with FVIII replacement therapy, because activated FVIII has a greater affinity for FIXa and FX than emicizumab and a synergistic effect is not expected (83, 94). However, patients with inhibitors can be treated with rFVIIa in normal dosage, and if not available or not effective, with very low dose aPCC with careful thrombin generation monitoring as multiple patients have developed thrombosis after administration of aPCC (dosed >200 IU/kg/day) in combination with emicizumab (78).

Anti-TFPI treatment enhances the initiation phase of coagulation by inhibiting the shutdown effect of TFPI resulting in a prolonged TF-FVIIa activity, leading to an increased activation of FX and eventually thrombin (95). The now discontinued agent BAX-499 already showed improved thrombin generation in hemophilic plasma (96). Additionally, spiking studies with two different anti-TFPI antibodies (marstacimab and befovacimab) both increased thrombin generation to a level that was approximately equal to a FVIII activity level of 40% (97, 98). Most pharmacodynamic studies are performed with concizumab which showed a dose-dependent increase in thrombin generation, even in plasma of healthy volunteers (79, 99). Afterward, pharmacodynamic monitoring was used to determine the eventually investigated dose of concizumab (100). Patients who reached the prespecified concentration of concizumab (>100 ng/ml) showed normalization of thrombin generation (101). Lastly, concomitant therapy of concizumab with aPCC, rFVIIa, and FVIII showed additive effects, instead of exponentially effects such as between aPCC and emicizumab (102). Therefore, concizumab can be combined with other treatment modalities, but dosages should be adjusted and monitored with TGAs to provide safe and effective therapy.

Antithrombin lowering can be established with fitusiran, an anti-sense oligonucleotide directed against antithrombin mRNA, leading to decreased inhibition of coagulation. Studies showed increasing amounts of thrombin generation with further reducing antithrombin with reaching near normal levels of thrombin generation when antithrombin was >75% reduced (80, 81). Comparable with concizumab, fitusiran combined with aPCC or rFVIIa had additive effects on thrombin generation (103).

Thrombosis

The TGA can be used in the analysis of patients with thrombosis, for example to analyze the prothrombogenic phenotype in patients with (recurrent) VTE (104), and to monitor treatment with anticoagulant therapy (for most important findings, see **Table 1**).

Analysis of thrombotic tendency

First and recurrent venous thrombo-embolism

Venous thrombo-embolism consists of pulmonary embolism and deep vein thrombosis and is common in the general population. Patients are treated with anticoagulants to prevent further progression of the thrombus and preventing recurrent thrombosis. However, the ideal duration of anticoagulation in the individual patient is unknown and decisions about stopping/continuing anticoagulation were made on clinical characteristics and patient preferences. Possibly, the TGA could help indicate which patient has a hypercoagulable phenotype and has a high risk for first or recurrent VTE. This is investigated in multiple studies (105–114). The first indication that thrombin generation could influence VTE recurrence was with a RCT in which D-dimer level measured one month after discontinuation was used to indicate prolonged anticoagulation. Patients with an elevated D-dimer level who restarted anticoagulation had a significant lower chance of recurrent VTE than patients without anticoagulation (115). Furthermore, it was shown that patients with VTE have significantly higher ETP values than controls without VTE (105, 106, 109). One study found that thrombin generation was higher in individuals with an additional risk factor for the development of VTE than patients with an idiopathic VTE (105). Another study, however, reported higher thrombin generation in patients with idiopathic VTE compared to those with provoked VTE, even after correction for FVIII and D-dimer levels (112). Furthermore, multiple studies showed that addition of thrombomodulin to the TGA was able to magnify the differences found between patients and controls (105–107).

The risk of recurrent VTE can be estimated with TGA with increased hazard ratios (HR) ranging from 1.6 to 4.0 for increased ETP and subsequent recurrent VTE (107, 108, 113). The HR for recurrent VTE based on thrombin peak height was even 4.6 in one study (107). One study could not establish an increased risk for recurrent VTE (HR 1.1) when elevated ETP was used to distinguish patients and controls (109). This different conclusion can be explained by the study design, as the last study was a case-control study, while all others were prospective cohort studies. Interestingly, a cohort study also showed that patients with low thrombin generation had a lower risk of recurrent VTE (HR 0.40) (111). Thereby confirming the risk association between thrombin generation and the risk of recurrent VTE.

Lastly, patients with cancer have a high risk of VTE development. Ay et al. performed TGA in 1033 patients with

TABLE 1 Summary of thrombin generation assay characteristics and findings in bleeding and thrombotic diseases.

Clinical scenario	TGA conditions*	TGA parameter	Important findings	References
Bleeding disorders				
Diagnosis of bleeding of unknown cause	PPP, low	Lag time, TPH, TP	Lower thrombin generation in patients with bleeding of unknown cause compared to normal controls.	(31, 33–36)
Clinical phenotype of bleeding disorders	PPP, low PRP, low	TPH, TP TPH, TP	HA patients with severe bleeding have lower thrombin generation despite equal FVIII activity level; thrombin generation is lower in RBD patients with bleeding. FXI deficiency bleeding phenotype can be distinguished with high diagnostic accuracy.	(40–44, 49)
Treatment monitoring				
Factor concentrate	PPP, low	TPH, TP	Bleeding patients with prophylactic therapy have lower thrombin generation than not bleeding patients; thrombin generation is suggested to be used for individualized prophylactic treatment schemes.	(55–66)
Bypassing agents (BPA)	PPP, low	TPH, TP	Monitoring of the effect of BPAs on hemostasis and selecting the bypassing agent with the most efficacy in the individual patient.	(69–77)
Emicizumab	PPP, low	TPH, TP	Emicizumab restores thrombin generation to 20-30% of normal; possibility to monitor the effect of combination therapy with emicizumab and FVIII concentrate or BPAs.	(84–93)
Anti-TFPI	PPP, low	TPH, TP	Patients reaching the prespecified concentration of concizumab had normalization of thrombin generation; possibility to monitor concomitant use of FVIII concentrate or BPAs.	(99–102)
Antithrombin antisense	PPP, low	TPH, TP	Normalization of thrombin generation when antithrombin was > 75% reduced; monitoring of combined therapies.	(80, 81, 103)
Thrombotic disorders				
Diagnosis of first and recurrent VTE	PPP, high	TPH, TP	Higher rates of first and recurrent thrombosis in patients with increased thrombin generation; lower risk of recurrent thrombosis with low thrombin generation.	(105–114)
Diagnosis of arterial thrombosis	PRP, high	Lag time, TPH, TP	Some indications that thrombin generation is increased in coronary artery disease, for ischemic stroke conflicting evidence.	(118–132)
Antiphospholipid syndrome	PPP, high	Lag time, TPH; TP	Normalized thrombin peak height/lag time ratio can identify patients with antiphospholipid syndrome; identification of patients with suboptimal treatment according to thrombin generation despite adequate INR.	(135, 137–146)
Anti-thrombotic therapy				
Heparinoids	Variable, high	TPH, TP	Tinzaparin provides greater thrombin generation reduction compared to other heparinoids; anti-Xa measurement possibly less indicative for hypercoagulable state.	(150–155)
Vitamin K antagonists (VKA)	PPP, high	Lag time, TPH, TP	INR has a correlation with lag time; lower thrombin generation is associated with bleeding in VKA treated patients.	(156–159)
DOACs	PPP, high	TPH, PT	Thrombin generation is already inhibited at a low DOAC concentration; reversal with BPAs can be monitored with thrombin generation; low correlation between anti-Xa concentration and thrombin generation.	(162–180)
Antiplatelet therapy	Variable#	Velocity of thrombin generation, TPH, PT	PPP is less sensitive for the measurement of thrombin generation then PRP; velocity of thrombin generation, TPH and TP are most affected by antiplatelet therapy.	(181–184)

*Thrombin generation assay characteristics that give the best results in this condition or are used by most studies in this area. TGA determined in platelet poor plasma (PPP) or platelet rich plasma (PRP), with low (usually 1 pM) or high (usually 5 pM) tissue factor as starting reagent.
#In studies reporting TGAs with antiplatelet therapy PPP and PRP are used, with both low and high concentrations of tissue factor.
BPA, bypassing agent; DOACs, direct oral anticoagulant; FVIII, factor VIII; FXI, factor XI; INR, international normalized ratio; PPP, platelet poor plasma; PRP, platelet rich plasma; RBD, rare bleeding disorder; TFPI, tissue factor pathway inhibitor; TGA, thrombin generation assay; TP, thrombin potential; TPH, thrombin peak height; VTE, venous thrombo-embolism; VKA, vitamin K antagonist. Lars L. F. G. Valke: LV Sanna Rijpma: SR Danielle Meijer: DM Saskia E. M. Schols: SS Waander L. van Heerde: WH.

various types of solid tumors and found a HR of 2.1 for the development of a VTE event in patients with the highest quartile of thrombin generation. Incidence of VTE in the first 6 months was 11% in this quartile, compared to 4% in patients with lower thrombin peak height (116). Therefore, it can be concluded that thrombin generation might be a useful tool to predict first and recurrent VTE incidence, in patients with idiopathic and provoked VTE, and in patients with a

malignancy. However, it should be noted that absolute cut-off values of thrombin generations parameters are not possible, because a large overlap in thrombin generation profiles exists between VTE patients (114).

Arterial thrombosis

In contrast to VTE, the role of thrombin generation measurement is less clear in patients with arterial thrombosis. Arterial thrombosis is a leading cause of death worldwide and consists of coronary artery disease (CAD) and ischemic stroke. It is a complex interaction between the long lasting process of atherosclerosis of the main arteries, in combination with acute rupture of an atherosclerotic plaque that provokes thrombus formation at the site of injury. Only if the thrombus limits blood flow to the affected organ, symptoms can be reported by the patient. Since atherosclerosis and inflammation are strongly linked to each other and inflammation has a role in thrombin generation, the exact relationship between arterial thrombosis and thrombin generation is hard to establish and conflicting evidence is reported (117).

It is shown that patients with CAD have higher thrombin generation during an acute myocardial infarction and during the chronic phase, compared to patients with stable disease (118). This suggests that these patients are in a more hypercoagulable state and are more prone to arterial thrombosis (119). Increased thrombin generation parameters (thrombin potential and thrombin peak height) are often described in patients with acute MI or CAD (118, 120–122), but also a prolonged lag time is described (123). However, other studies describe a more U-shaped association between thrombin potential and CAD (124, 125). The association between enhanced thrombin generation and arterial thrombosis was further investigated in a case-control study with patients with an in-stent thrombosis after myocardial infarction. Here again, it showed that patients had higher thrombin generation compared to controls who did not have in-stent thrombosis (126). Furthermore, patients with residual detectable thrombin generation after percutaneous coronary intervention (PCI) despite optimal antiplatelet and periprocedural anticoagulant therapy had a higher risk of cardiovascular death (127).

For ischemic stroke, evidence is less clear. In one study, young stroke patients had an increased thrombin potential in PRP, while the association was not found in PPP (128). Multiple, smaller studies did not show an association between thrombin generation and adverse events (129, 130). These studies could be hampered by their sample size, since in one cohort study of more than 9,000 persons, a significant association was found between thrombin generation and the development of ischemic stroke. This study suggests that ischemic stroke could be prevented by diminishing the hypercoagulable state in these patients (122). On the other hand, another prospective cohort study found a significant inverse relationship between thrombin generation and the development of stroke (121).

In summary, the relationship between thrombin generation and arterial thrombosis is not readily defined. Thrombin generation in patients with CAD is increased in most studies, but the effect is only substantial. In patients with ischemic stroke, the evidence is even less clear-cut. These differences can be explained by study design or study population (117). Furthermore, the influence of traditional risk factors for cardiovascular disease on thrombin generation cannot be excluded. For example, obesity has been shown to increase thrombin potential (131). Also, another study showed that the concentration of apolipoprotein C-III was an independent risk factor for CAD, but also that it was associated with thrombin peak height and thrombin potential (132). Still, it is established that higher thrombin potential is associated with increased total mortality (131). Further research in this field should elaborate on thrombin generation in both PPP and PRP, because thrombin generation in arterial thrombosis is an interplay between vessel wall, platelets and coagulation factors.

Antiphospholipid syndrome

The antiphospholipid syndrome (APS) is characterized by obstetric complications and/or arterial/venous thrombosis in combination with typical antiphospholipid antibodies (aPL antibodies: lupus anticoagulant (LAC) and/or anti-β2 glycoprotein I (aβ2GPI) and/or anti-cardiolipin (AC)) measured twice with at least 12 weeks in between (133). Major assay heterogeneity and lack of standardization cause problems with the diagnosis of APS (134). Also, non-pathogenic aPL antibodies can be encountered, for example in the presence of certain infections or medication. Furthermore, not all carriers of aPL antibodies develop thrombo-embolic complications. In this regard, the TGA may play an important distinguishable role (134).

In the diagnostic process, a chromogenic TGA was able to detecting all three aPL antibodies and could even distinguish between APS antibodies and antibodies arisen from transient causes, such as infections. However, this assay used purified antibodies and NPP, making it not available for use in routine clinical practice (135). Multiple groups have shown that aPL antibodies cause a lag time prolongation in the TGA, potentially due to shielding of the exogenous added phospholipids (136, 137). However, in these patients, thrombin peak height was increased as well, which led to the proposition of the use of the normalized peak height/lag time ratio (PH/LT-ratio) (138). This ratio was able to detect LAC antibodies with high sensitivity, even in anticoagulated patients (139). But additional research is necessary to establish that increased thrombin generation is due to APS instead of other causes, since increased thrombin generation is also seen in patients with VTE (as described in section "First and recurrent venous thrombo-embolism").

Multiple studies have shown that the increased thrombin generation observed in patients with APS is mainly due to increased activated protein C (APC) resistance (137, 140, 141).

This APC resistance was associated with thrombotic events (139–143), and was even incorporated in a ratio that could predict thrombosis over time (144). Lastly, TGAs can also be used to determine the degree of anticoagulation in patients with APS (145, 146). It even showed that a subgroup of patients had increased thrombin generation despite adequate international normalized ratio (INR) values. Thereby it is a possible tool to identity APS patients with an ongoing prothrombotic state despite therapy with vitamin K-antagonists (VKAs) (145). In the next paragraph, anticoagulation monitoring with TGAs will be further described. Thereby, it can be concluded that TGAs can be used in combination with classic APS assays to provide a more detailed impression of the hypercoagulable state of patients with thrombosis due to APS. Furthermore, monitoring of anticoagulation in these patients can be helpful, as recurrent thrombosis is common in APS patients (147).

Treatment with anticoagulant therapy

Arterial and venous thrombo-embolic disorders are treated with different kinds of anticoagulant therapies, depending on the indication and patient characteristics. For most of these treatments, some kind of test to monitor the effect exists in the laboratory. However, mostly this encompasses a part of the coagulation cascade, like anti-Xa monitoring for heparinoids or LMWH, and it does not take hyper- or hypocoagulability of the patient into account (148). This part of the review will focus on anticoagulant therapy with heparinoids, VKA, DOACs and lastly antiplatelet therapy.

Heparinoids

Heparin treatment can be divided in unfractionated heparin (UFH) and low molecular weight heparin (LMWH) therapy. UFH treatment needs to be monitored by measurement of APTT and/or anti-Xa, while treatment with LMWH is often fixed-dosed or weight-based dosed (149). However, LMWH is sometimes monitored with anti-Xa determination at the extremes of the weight spectrum (e.g., cachexia and morbid obesity) and in patients suffering from renal insufficiency. With anti-Xa monitoring, it appears that some patients show widely different anti-Xa activity levels with the same dosage, therefore, thrombin generation monitoring could be of interest in patients treated with UFH or LMWH (149).

The anticoagulant effect of UFH is comparable with different kinds of LMWH in spiked PRP (150). This study showed that tinzaparin had greater thrombin generation inhibitory effects compared to UFH and other LMWHs at the same anti-Xa activity level (150), which was confirmed in a second study that compared enoxaparin with tinzaparin (151). Moreover, it was shown that fondaparinux, a synthetic pentasaccharide which inhibits Xa formation via antithrombin, had less inhibitory effect on thrombin generation if compared to LMWH (150, 152).

Thrombin generation in the presence of LMWH was also measured in some specific populations. It is known that thrombin generation increases during pregnancy. In one study, healthy pregnant women, pregnant women with mild (e.g., heterozygous factor V Leiden) and severe thrombophilia (e.g., homozygous factor V Leiden) were followed each trimester with thrombin generation measurement. In women with severe thrombophilia, thrombin generation increased more than in women without thrombophilia (153). Prophylactic LMWH dosage inhibited thrombin generation. However, in the third trimester, thrombin generation was significantly elevated despite stable anti-Xa activity levels over time (153). Suggesting that pregnant women are in a hypercoagulable state despite fixed prophylactic LMWH therapy. This effect was also shown in morbidly obese pregnant women, which showed higher thrombin generation parameters compared to normal weight pregnant women. Interestingly, the authors showed that a weight-based prophylactic LMWH dosage led to significant lower ETP values compared to standard-dosed LMWH (154). Lastly, the TGA was able to detect a hypercoagulable state in patients with cancer and showed normalization of thrombin generation whilst patients were on LMWH therapy (155).

Vitamin K antagonists

Before the introduction of DOACs, VKA were the main oral anticoagulants used. Dosing of VKA was personalized by measurement of the INR with subsequent dosage adjustments because multiple factors, like diet and genetic variants, influence the effect of VKA. Bleeding is the main risk of anticoagulation, therefore the goal is to keep the INR in a prespecified range. However, the INR only gives an impression of procoagulant factors, while anticoagulant factors are also influenced by VKAs. Therefore it would be interesting to know if VKAs could also be monitored with TGA.

Thrombin generation in the VKA treated patient showed a significant correlation with INR values, especially for lag time (156). In another study, however, some patients showed persisting thrombin generation despite adequate INR values (145), possibly indicating that they were still prone to recurrent VTE. When warfarin was compared with rivaroxaban, a DOAC, it appeared that overall thrombin generation parameters were comparable. Rivaroxaban exhibited slightly longer lag time, time to thrombin peak and lower thrombin peak height, while warfarin showed a lower ETP (157). However, in a study investigating an APS patient, rivaroxaban showed higher thrombin generation compared to warfarin and enoxaparin (146).

Interestingly, in a prospective study investigating bleeding episodes in patients using VKAs, it appeared that patients with bleeding had significant lower thrombin peak height and ETP values measured with whole blood TGA, compared to patients who did not bleed. The patients with bleeding also had higher HAS-BLED scores, indicating that both whole

blood TGA and HAS-BLED score showed an association with bleeding (158). In another prospective cross-sectional study ETP was lower in warfarin treated patients who presented at the emergency department with bleeding, compared to warfarin treated patients who presented with another medical emergency, while INR was within target range in both groups (159).

Direct oral anticoagulants

Direct oral anticoagulants can inhibit thrombin (dabigatran) or FXa (apixaban, edoxaban, and rivaroxaban) and are given in a fixed dosing regimen, either once daily (edoxaban and rivaroxaban) or twice daily (apixaban and dabigatran). The main advantage of DOACs over VKAs is that monitoring of anticoagulation is not required (160). However, in some instances, for example in case of bleeding, recurrent thrombosis, or renal insufficiency, monitoring the effect of anticoagulation with DOACs can be of interest. In this regard, the anti-IIa or anti-Xa can be useful, but only gives an impression of the effect of the drug and not of the overall hemostatic capacity of the patient (161). The effect of DOACs on thrombin generation has been studied quite extensively, with *in vitro* studies as well as with plasma from patients using DOACs.

The *in vitro* studies showed that thrombin generation is hampered by DOACs (162–170). However, the parameters that are affected differ with the kind of DOAC. For example, dabigatran resulted in an increased lag time, while thrombin peak height and ETP remained relatively normal (162–164). On the other hand, presence of FXa inhibitors (apixaban, edoxaban and rivaroxaban) was shown by an increased lag time, but also an increased time to thrombin peak, with additional decreased thrombin peak height and ETP (163–167). Most studies that compare different DOACs, have shown that rivaroxaban has a stronger inhibitory effect on thrombin peak height and ETP compared to apixaban and edoxaban (163, 164). Furthermore, *in vitro* spiking plasma of pediatric and adult patients with edoxaban showed an equal inhibitory effect on thrombin generation among different age groups, except children <2 years of age, who had a stronger inhibition of thrombin generation at the same concentration of edoxaban (169).

Concerning thrombin generation and the use of DOACs, in healthy volunteers taking dabigatran, rivaroxaban and apixaban on different occasions, the same parameters were affected as with *in vitro* measurements (171). Dabigatran only increased lag time, while apixaban and rivaroxaban both inhibited thrombin peak height and ETP (171–174). Further studies showed that apixaban and rivaroxaban have a non-linear inhibitory function for thrombin generation. This indicates that most thrombin generation inhibition occurs at low anti-Xa concentrations (i.e., with a low concentration of DOAC, thrombin generation is still inhibited) (175, 176). Therefore, the authors further investigated how much thrombin was generated 12 h after intake of a DOAC. Thrombin generation was still inhibited at this time point, suggesting that an urgent surgery was not possible when

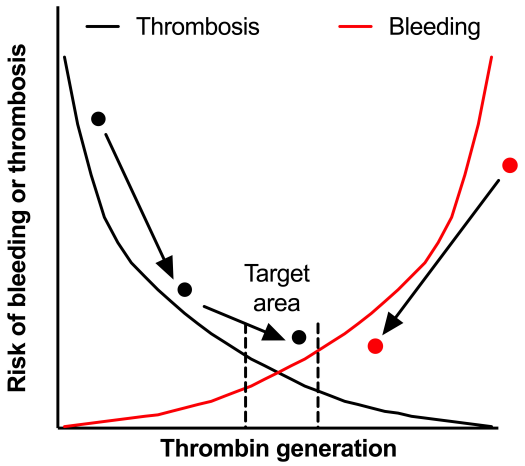

FIGURE 2
The hemostatic balance. The hemostatic balance is depicted as the risk of thrombosis (black line) and the risk of bleeding (red line), which is dependent on thrombin generation (on the x-axis). In the target area, both the risk of thrombosis and the risk of bleeding are acceptably low, but can still occur in an individual patient in certain circumstances. Two patients are shown in the figure, the black dot represents a patient with a venous thrombo-embolism during an hypercoagulable state. With treatment the hypercoagulable state is diminished, but the patient experiences a second thrombotic event while on adequate anticoagulant therapy. By intensifying anticoagulant treatment, this patient reaches the target area. On the other side of the curve, the red dot represents an hemophilia A patient with a high bleeding rate. After starting prophylactic therapy with factor VIII concentrate, the bleeding phenotype improves, but doesn't reach the target area. However, for this patient the reduction in bleeding is acceptable, while intensifying treatment could lead to overshoot to a risk of thrombosis. In both patients, monitoring with TGAs could identify the target area better. This could have prevented the second thrombosis in the patient represented by the black dot as the hypercoagulable state was recognized earlier.

thrombin generation is used as surrogate marker for hemostatic normalization (177). Furthermore, plasma levels of DOACs did not correlate with the extend of thrombin generation inhibition (178, 179). Therefore, thrombin generation measurement to provide an individual thrombin generation profile could be of more importance in a patient presenting with an acute bleeding or in need of urgent surgery than measuring DOAC anti-Xa activity level.

Lastly, several studies have investigated *in vitro* the effects of DOAC reversal therapy. These studies show that a TGA can help to establish correction of thrombin generation after addition of reversal agents (162, 166, 180). This is of major importance because other laboratory assays that do not measure hemostasis as a whole and cannot be used (i.e., anti-Xa assays or factor level activity assays) (161).

Antiplatelet therapy

Even though antiplatelet therapy, like aspirin and clopidogrel, do not affect coagulation factors, the effect of

these therapies on thrombin generation were studied in PRP as well as PPP. A case-control study showed that patients with CAD followed by PCI who were treated with standard dosage of dual antiplatelet therapy with aspirin and clopidogrel had significant longer time to thrombin peak, decreased thrombin peak height and ETP than controls in PRP (181). Velocity of thrombin generation was most impaired in patients. These differences in thrombin generation parameters were not found in PPP but only in PRP suggesting the importance of platelets (181). In a longitudinal study investigating thrombin generation after ischemic stroke, it was shown that aspirin in combination with dipyridamole significantly decreased thrombin peak height and ETP, while aspirin monotherapy and clopidogrel did not significantly change thrombin generation compared to baseline measured in PPP and not in PRP (182). Another study showed that platelet reactivity, measured with different platelet-activity assays, did not correlate with thrombin generation, measured in PPP (183). These studies show the importance of measurement thrombin generation in PRP, because in PPP it is less sensitive to assess the effects of these drugs.

In a study by de Breet et al., thrombin generation was measured in PPP one and six month after PCI for CAD. Patients were followed for one year to assess bleeding. It appeared that patients with bleeding had a significant lower thrombin peak height, ETP and velocity of thrombin generation at 1 and 6 months after PCI compared to patients without bleeding. Suggesting that performing TGA is possibly to identify patients with clinical relevant risk for bleeding episodes whilst using dual antiplatelet therapy (184).

Thrombin generation assays to personalize medicine

Personalized medicine

Personalized medicine is becoming increasingly important in research and clinical practice and aims that "medial decisions, practices, interventions, and/or therapeutic agents are being tailored to the individual patient, based on their predicted response to treatment or risk of disease" (185). In other words, it aims to adjust treatment to each patient individual needs and preferences.

The processes of thrombosis and hemostasis are often depicted as a balance or two crossing lines (**Figure 2**). The lines represent the chance of bleeding or thrombosis, the pathological outcomes, which is probably more represented by thrombin generation in the individual patient. Even within the small target area, patients are still at risk for bleeding and thrombotic events, even though this risk is smaller than at the extremes of the curve. This can for example be seen in a patient with thrombosis (the black dot in **Figure 2**) due to a hypercoagulable state. With the use of anticoagulation, for example VKA with a target INR of 2–3, the risk of VTE recurrence is lowered, but some patients can still be hypercoagulable while having an INR within the target range (145). Therefore this patient can experience a recurrent VTE and only after intensification of the INR target range to 3–4, the patient's hemostatic balance is within the referred range. In this example, VKA therapy and monitoring can be seen as a form of personalized medicine, because the number of tablets is dependent of the measured INR value. However, PT-based INR monitoring is highly artificial (due to a high TF concentration) and anticoagulant factors, like APC resistance, are not part of the INR test. Therefore INR is not really monitoring the hemostatic balance, while TGAs are more physiological and are expected to better reflect the patients hemostatic potential.

Because the INR can be sub- or supratherapeutic, with an accessory risk of thrombosis or bleeding, respectively, DOACs were developed. Since DOAC therapy has a standard dosage regimen based on evidence-based medicine, the manufactures advocate that monitoring is not required. However, patients can also be over- or underdosed. This is illustrated by the percentage of bleeding and recurrent thrombotic events in the DOAC trials (6), thereby indicating that for the main population, the dosage of the DOAC is correct, but for a number of patients it still results in either bleeding or thrombosis. TGAs, however, could give an impression of the hemostatic balance of the individual patient. This was illustrated in a case report in which the dosage of rivaroxaban was adapted based on thrombin generation results (186).

In hemophilia treatment, personalized medicine is becoming the standard of care. Prophylactic therapy with factor replacement therapy decreases the bleeding phenotype from a regular and spontaneous bleeder to become a mild bleeder (red dot in **Figure 2**). By intensifying prophylactic treatment, either by increasing the dosage or shortening the interval, the treatment can be personalized to a situation in which the patient has even less bleeds. However, by intensifying treatment, the chance of thrombotic disease may increase and costs will rise temporarily. Global assays may overcome this as it is expected that these assays better reflect the hemostatic balance. Measurement of thrombin generation can be combined with FVIII activity level and this could be used as the basis for an individualized treatment scheme, with the help of a population based PK-PD model (63, 66). However, this strategy is not yet tested in daily clinical practice.

Controversies and limitations

Despite the overwhelming amount of evidence in favor of implementing TGAs in thrombotic and bleeding disorders

to personalize medicine, some controversies and limitations remain to be addressed. Four important points need to be addressed, of which two are related to the methodology of the TGA and two regarding the monitoring of the hemostatic balance.

First, different kinds of (commercial) platforms exist to determine thrombin generation. In general, all platforms use the same method, but with slightly different concentrations of reagents, which are often undisclosed. This leads to varying amounts of generated thrombin with associated variating normal values. This problem could be targeted by normalizing the TGA parameters with normal pooled plasma (NPP). Even though it is assumed that NPP should approximately contain a normal activity level of all coagulation factors, there is still difference in thrombin generation with different NPP. This was nicely illustrated by a study that investigated the coefficient of variation of three plasmas with different coagulation profiles. Despite using the same TGA, results differed more than the predefined 25% and normalizing the results with the laboratory's own NPP did not improve variation (187). Therefore, before the TGA can be compared across studies and can be used in daily clinical practice, the methods and materials used should be harmonized.

Second, performance of the TGA is time consuming because of preparation of plasma and the duration of the assay itself. For research purposes this is not a problem, as the TGA is often performed in batches to minimize variation. However, in clinical practice, the need of TGA determination can be urgent, for example, as mentioned in case of bleeding in a HA patient treated with emicizumab, or a patient using a DOAC. The whole blood viscoelastic tests (e.g., ROTEM) can be determined directly and results are available within an hour. Therefore, the determination of the TGA should be faster and possibly applicable as a point-of-care test (POCT). Already progress is being made to develop a POCT TGA which can be utilized at home for monitoring hemophilia patients with a bleed or at the emergency services to screen for coagulation defects in multi-trauma patients.

Thirdly, the target range of TGAs on the hemostatic balance is not known. This means that normal values of a larger healthy population are known, but it is unknown if this range is also the target range to prevent bleeding and thrombosis for an individual patient. Furthermore, each individual has a certain amount of thrombin generation during stable situations. However, during a thrombotic event, thrombin generation can be higher compared to the normal situation due to an intercurrent event which may has caused the thrombosis. The question is to what extent the patient should be treated; to the amount of thrombin generation before the event (if this is known), or to a predefined target based on large evidence based studies. In both cases, the patients treatment is according the principals of personalized medicine. However, only in the first scenario the patient is treated according to its own hemostatic need, which probably prevents bleeding due to the right amount of anticoagulation.

Fourth, the TGA is just one parameter whereas the hemostatic balance is orchestrated by the vessel wall, platelets, coagulation factor and fibrinolysis. The ultimate goal would be a global assay that measures these parameters simultaneously.

Future perspective and conclusion

Thrombin generation assays can play an important role in the assessment of bleeding and thrombotic diseases, like diagnosis and prognosis of coagulation disorders, both inherited and acquired, as well as monitoring of the treatment of these diseases. However, a number of important items need further attention. The most important is the comparison and standardization of different TGA platforms with uniform reporting of the (local) thrombin generation reference values and normalization to NPP. Furthermore, most of the studies described in this review contain only small numbers of patients and are often monocentric. Larger studies with the use of standardized TGA systems could help for better adaptation of the assay in clinical practice. Also, serial determination of thrombin generation during health and disease (bleeding, thrombosis, and/or hypercoagulable states like cancer) in an individual patient will help understanding the variation in thrombin generation over time. This will give insight in the determination of the target range for treatment of bleeding and thrombotic disorders. Lastly, rapidly available, POCT TGA testing are promising to determine the hemostatic balance of an individual patient with acute hemorrhage due to acquired or congenital bleeding disorders.

In conclusion, TGAs are a versatile tool to measure the coagulation cascade as a whole in bleeding and thrombotic diseases. Since it measures the individual patients hemostatic balance, it can be used to personalize medicine of patients with bleeding disorders, thrombosis and monitoring (anti)hemostatic therapy (see **Table 1**). Results of recent research show that personalized medicine based on TGAs is most promising for patients with HA. Especially with the emerging non-factor replacement therapies and concomitant usage of therapies, hemostatic monitoring on an individual patient level is essential. The personalization of therapies could ultimately lead to tailoring the treatment of these disorders to needs of the patients without exposing them to unnecessary bleeding or thrombotic risks. However, before utilization in clinical practice, some important hurdles should be taken.

Author contributions

LV and SR wrote the first draft of the manuscript. DM, SS, and WH critically revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

WH received unrestricted grants from Bayer, Shire, Novo Nordisk, and CSL Behring. WH is the founder and CSO of Enzyre BV, a Radboudumc spinoff company. SS received a research grant from Bayer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Intrinsic coagulation pathway-mediated thrombin generation in mouse whole blood

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Calibrated Automated Thrombography (CAT) is a versatile and sensitive method for analyzing coagulation reactions culminating in thrombin generation (TG). Here, we present a CAT method for analyzing TG in murine whole blood by adapting the CAT assay used for measuring TG in human plasma. The diagnostically used artificial and physiologic factor XII (FXII) contact activators kaolin, ellagic acid and polyphosphate (polyP) stimulated TG in murine blood in a dose-dependent manner resulting in a gradual increase in endogenous thrombin potential and peak thrombin, with shortened lag times and times to peak. The activated FXII inhibitor rHA-Infestin-4 and direct oral anticoagulants (DOACs) interfered with TG triggered by kaolin, ellagic acid and polyP and TG was completely attenuated in blood of FXII- (*F12*$^{-/-}$) and FXI-deficient (*F11*$^{-/-}$) mice. Moreover, reconstitution of blood from *F12*$^{-/-}$ mice with human FXII restored impaired contact-stimulated TG. HEK293 cell-purified polyP also initiated FXII-driven TG in mouse whole blood and addition of the selective inhibitor PPX_Δ12 ablated natural polyP-stimulated TG. In conclusion, the data provide a method for analysis of contact activation-mediated TG in murine whole blood. As the FXII-driven intrinsic pathway of coagulation has emerged as novel target for antithrombotic agents that are validated in mouse thrombosis and bleeding models, our novel assay could expedite therapeutic drug development.

KEYWORDS

thrombin generation, plasma contact system, factor XII, polyphosphate, CAT assay, diagnostics, coagulation, mouse whole blood

Introduction

The coagulation of blood culminates in the generation of thrombin, a protease that activates platelets and converts soluble fibrinogen to insoluble fibrin with subsequent polymerization and clot formation (1, 2). Kinetics of thrombin generation (TG) are highly complex and orchestrated by a delicate network of thrombin producing (procoagulant mechanisms) and terminating (anticoagulant mechanisms) reactions. Calibrated Automated Thrombography (CAT) allows for real-time monitoring of active thrombin concentrations in clotting plasma that reflect the complexity of generation and neutralization of the protease (3). CAT is based on the conversion of a thrombin-specific fluorogenic substrate, whose signal is normalized to an internal standard. This setup allows for highly sensitive monitoring of levels of the active enzyme in patient plasma samples in a diagnostic setting. The resulting thrombogram curve represents stimulus-dependent thrombin levels over time, providing information on (i) the time that elapses until TG is initiated (lag time), (ii) the time to maximal TG (time to peak), (iii) the maximal level of thrombin formed (peak thrombin), and (iv) the total amount of thrombin generated over time (endogenous thrombin potential, ETP, Supplementary Figure 1). The latter is often referred to as "area under the curve" and reflects the calculated time integral of thrombin concentrations in stimulated plasma.

TG involves complex activation of multiple clotting factors on membrane structures that expose phospholipids (PL), especially phosphatidylserine. As TG measurements are mainly performed with platelet-poor plasma (PPP) in the absence of blood circulating cells, addition of a synthetic PL mixture is required to provide a micelle-like surface structure that mediates coagulation factor surface recruitment and, by lateral diffusion, complex formation of these factors. In addition to the coagulation system that is analyzed by CAT in PPP, roles of platelets in TG can be studied using CAT analysis in platelet-rich plasma (PRP) (3). As such, PRP allows for analysis of the crosstalk between (activated) platelets and the coagulation system (primary and secondary hemostasis, respectively). However, the effect of other blood cells that have a major role in coagulation reactions *in vivo*, such as leukocytes and erythrocytes, on TG remains largely unidentified. Indeed, analysis of TG in whole blood samples is challenging as erythrocytes tend to sediment rapidly, leaving the ratio of fluid vs. cellular/membranous compartments undefined. Whole blood TG analysis can be further confounded by the release of hemoglobin from preanalytical hemolysis that may interfere with the fluorescent signal. As a result, TG assays developed for human whole blood are prone to high data variability (4), require extensive operating experience (5) and are unable to analyze more than one sample simultaneously (6, 7). These limitations can be circumvented by an experimental setup that enables continuous shaking of the blood sample throughout the TG measurement (8).

Since mouse models are an important tool for studying blood coagulation activation under physiological and pathological conditions, fluorogenic assays have also been developed to measure TG in mouse whole blood, where filter paper disks are typically used to prevent erythrocyte sedimentation. However, the usage of filter paper produced inaccurate readouts as the filter surface triggered contact activation of coagulation factor XII (FXII) (9, 10), which initiates TG. FXII is an essential part of the plasma contact system together with plasma prekallikrein (PK) and the non-enzymatic cofactor high molecular weight kininogen (HK). FXII circulates as a zymogen in blood and is activated by binding ("contact") to negatively charged surfaces (11, 12), inducing a conformational change that leads to its conversion to the active protease FXIIa. Subsequently, FXIIa proteolytically cleaves PK forming activated plasma kallikrein (PKa), which in turn activates additional FXII zymogens in a positive feedback loop leading to amplified FXIIa production (fluid-phase activation). Sequential limited proteolysis of the FXIIa substrate factor XI (FXI), followed by factor IX and factor X, is referred to as the intrinsic pathway of coagulation. As a result, thrombin is produced followed by the formation of fibrin clots. FXII deficiency protects from pathological clot formation *in vivo* and genetic ablation of *F12* in mice ($F12^{-/-}$) prevents arterial and venous thrombus formation without increasing the risk of bleeding (13, 14). Accordingly, $F12^{-/-}$ mice are protected from thromboembolic disorders including ischemic stroke (15), cancer-associated thrombosis (16, 17), pulmonary embolism (18), and sepsis-mediated thrombosis (19).

Importantly, TG measurements in mouse whole blood have provided more detailed insights into the role of erythrocytes in FXII contact activation and its contribution to hyper- and hypo-coagulable states in experimental models. Here, we developed a modified CAT-based method (8) to precisely assess plasma contact system-triggered TG, in which samples were continuously mixed throughout the measurement to alleviate quenching of the fluorescence signal by red blood cells. Our data indicate that TG is largely reduced in the presence of direct oral anticoagulants (DOACs) and inhibitors targeting plasma contact system proteins, and is defective in whole blood from $F12^{-/-}$ and FXI-deficient ($F11^{-/-}$) mice. Therefore, our assay represents a promising new technique to study the impact

Abbreviations: CAT, Calibrated Automated Thrombography; ETP, endogenous thrombin potential; TG, thrombin generation; Inf-4, recombinant human albumin-tagged (rHA)-Infestin-4; PK, plasma prekallikrein; PL, phospholipids.

of aberrant contact system protein activity on TG in mouse whole blood.

Materials and methods

Reagents

Kaolin was obtained from Sigma Aldrich. The activated partial thromboplastin time (aPTT) reagent Dade® Actin® FS containing ellagic acid and soybean phospholipids and Pathromtin SL® were purchased from Siemens Healthcare Diagnostics. FXII contact activators STA® - C.K. Prest® and STA® - Cephascreen® were obtained from Diagnostica Stago (Asnières, France), while SynthAFax®, SynthASil® and APTT SP reagent were purchased from IL Werfen (München, Germany). The following synthetic polyphosphates with different lengths were obtained from ICL Pharmaceuticals: ammonium polyphosphate (P42, #2 2846), ammonium polyphosphate (P30, #2 2840), sodium polyphosphate (P75, #7 9990), sodium polyphosphate (P70, #7 1480), sodium polyphosphate (P68, #7 1480). Dornase alfa, the recombinant human DNaseI (Pulmozyme®), was purchased from Roche. FXII purified from human plasma was obtained from Haematologic Technologies. The FXIIa-specific inhibitor rHA-Infestin-4 was supplied by CSL Behring. Thrombin generation assays were performed with the fluorogenic substrate Z-Gly-Gly-Arg-AMC, purchased from Bachem Bubendorf, Switzerland. The thrombin calibrator (α2-macroglobulin-thrombin complex, α2M-T) and MP reagent (containing phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine at a ratio of 20:60:20) were obtained from Diagnostica Stago.

Collection of mouse blood samples and plasma preparation

Wild-type (WT), FXI-deficient ($F11^{-/-}$) and FXII-deficient ($F12^{-/-}$) mice were anesthetized with isoflurane prior to blood sampling from the retro-orbital plexus. Orbital puncture was performed with a glass capillary 2 cm in length. Blood was collected into 0.5 ml tubes containing 3.2% trisodium citrate (9:1 blood to citrate ratio), while the first drop was discarded. At the time of blood sampling, mice were 12–40 weeks old. All animals were kept according to national guidelines for animal care at the University Medical Center Hamburg-Eppendorf.

Blood samples were used for TG measurements within the first 4 h after blood collection. Mouse platelet-poor plasma was prepared by centrifuging blood (2,500 × g for 15 min, twice). The procedure for isolation of erythrocytes and subsequent preparation of samples with different hematocrit levels was performed as described by Ninivaggi et al. (5).

Real-time thrombin generation in mouse whole blood

The measurement of TG in mouse whole blood followed the protocol described by Wan et al. (8) with some major modifications. This assay was performed in 96-well round-bottom plates (Greiner Bio-One, #650101). Each well contained a final reaction mixture of 65 µl. First, 10 µl citrated mouse whole blood were added to the wells and then thoroughly mixed with 23 µl of the stimulator [kaolin (10 µg/ml), ellagic acid (0.2 µg/ml), polyphosphate (polyP, 2 µg/ml), aPTT reagents (final dilution of 1:20) or buffer (20 mM HEPES, 140 mM NaCl, 0.5% BSA, pH 7.35)]. The resulting mixture was immediately supplemented with 10 µl of the fluorogenic substrate Z-Gly-Gly-Arg-AMC (416 µM, reconstituted in 20 mM HEPES, 6% BSA, pH 7.35). Reaction mixtures were incubated for 10 min at 33 °C with orbital shaking. Finally, 22 µl $CaCl_2$ (6.1 mM) were automatically added to each well with a dispenser of a Fluoroscan Ascent fluorometer (Thermo Scientific, Waltham, USA) and thrombin formation was analyzed with the CAT method using the Thrombinoscope software package (version 5.0.0.742). To ensure continuous and thorough shaking throughout the 30-min measurement period, TG in 36 wells was monitored at 6-s intervals in each experiment. If added, the concentration of exogenous phospholipids (MP reagent, Diagnostica Stago) was 4 µM. Each condition/blood sample was analyzed in triplicates and for calibration measurements the stimulator was replaced by an α2-macroglobulin-thrombin complex with known activity (Diagnostica Stago). In some experiments TG was measured in the presence of the FXIIa-specific inhibitor rHA-infestin-4 (Inf-4, 500 µg/ml), DOACs or the kallikrein-blocking reagent aprotinin (200 KIU/ml), which were incubated with blood samples for 30 min prior to stimulation. PolyP (7.1 and 10 µg/ml, respectively) isolated from human embryonic kidney (HEK)293 cells was used to trigger TG, whereas an exopolyphosphatase (PPX) variant that lacks the domains 1 and 2 (PPX_Δ12, 500 µg/ml) was absent or added to polyP 1 h before stimulation.

Cell culture

HEK293 cells (ATCC: CRL1573) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with GlutaMAX™ (Life Technologies, #10566016), 10% (v/v) heat-inactivated fetal calf serum (Life Technologies, #10082147), 100 U/ml penicillin and 100 µg/ml streptomycin

(Life Technologies, #15140122). Cultures were incubated at 37 °C and 5% CO_2.

Extraction of polyP from HEK293 cells

Isolation of polyP was performed according to the procedure described by Nickel et al. (17), with minor modifications. In brief, confluent HEK293 cells were washed with PBS, detached from cell culture flasks by trypsinization, and centrifuged for 5 min at 200 × g. After washing the resulting cell pellet for three times with 50 mM Tris (pH 7.4), cells were incubated with sulfuric acid (0.3 M) and sodium chloride (3.5 M) for 5 min. The reaction mixture was adjusted to a pH of 7.4 using 2N sodium hydroxide followed by addition of 1 M Tris (pH 7.4) to achieve an osmolarity of 0.3 M. Cell lysates were first incubated with DNaseI (200 U/μl, in 3.5 mM $MgCl_2$) for 30 min at 37°C and secondly with proteinase K (20 mg/ml) for 1 h at 37°C. After removal of cell debris by centrifugation (1,000 × g, 10 min), sodium iodide was added to the lysate at a final concentration of 4.5 M. The mixture was transferred to Qiagen PCR purification columns (Qiagen, #28106). Following two consecutive washing steps [with 10 mM Tris pH 7.4, 1 mM EDTA, 100 mM NaCl, 50% (v/v) EtOH], polyP was eluted in 50 mM Tris (pH 7.4) and stored at −80°C until usage.

Analysis of polyP by urea-PAGE

Polyacrylamide gel electrophoresis (PAGE) with Tris/ boric acid/ EDTA (TBE) gels containing 15% (w/v) polyacrylamide and 7 M urea was performed to resolve polyP extracted from HEK293 cells. PolyP was visualized by negative staining with 4′,6-Diamidino-2-phenylindol (DAPI) as previously described (20). For staining, gels containing separated polyP were incubated with a fixative solution [25% (v/v) methanol, 5% (v/v) glycerol, pH 10.5] in the presence of DAPI (2 μg/ml) for 30 min with moderate shaking and without protection from ambient light. After washing the gels twice with fixative solution (without DAPI), the DAPI-bound polyP was photobleached using the ChemiDoc Imaging System (Bio-Rad). The images taken afterwards were analyzed using Image Lab software (Bio-Rad, version 6.1).

Expression and purification of PPX_Δ12

The expression and purification procedure of recombinant PPX_Δ12 was performed as described previously (21). In brief, DH5α competent *E. coli* cells were cultured at 37°C on an orbital shaker after transformation with plasmid DNA (pTrcHisB backbone) coding for PPX_Δ12. Induction of protein expression through the *pTrc* promoter was achieved by addition of 0.5 mM isopropylthio-β-D-galactoside (Sigma-Aldrich). After lysis of cells by sonication, protein purification was performed using an ÄKTA™ start FPLC system (GE Healthcare) connected to a 1 ml HisTrap FF crude column (GE Healthcare). Histidine-tagged proteins were eluted with a buffer containing 20 mM NaH_2PO_4, 500 mM NaCl and 500 mM imidazole. The buffer of the elution fractions was changed to PBS, pH 7.4.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) and are shown as mean values ± standard deviation (SD). Depending on results of normality tests, more than two groups were compared using one-way ANOVA followed by Dunnett's multiple comparisons test (parametric statistics) or the Kruskal-Wallis test followed by Dunn's multiple comparison (non-parametric statistics). Value levels of statistical significance are considered as follows: $P > 0.05$ (ns – not significant), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

Results

Establishment of a CAT assay in recalcified mouse whole blood

We aimed to systematically optimize experimental conditions for CAT analysis in mouse citrate anticoagulated whole blood following addition of the contact activator kaolin (a white clay component) upon recalcification. Increasing the blood volume (from 5 to 15 μl in a total reaction volume of 65 μl) augmented TG activity, reflected by a shortening in lag time and time to peak, and largely increased peak thrombin and ETP (Figure 1A, left panels). Coefficient of variation (CV) of 10 replicates was lowest for 10 μl whole blood (9.7%) (Figure 1A, right panel), compared to 5 μl (33.8%) and 15 μl (11.5%), respectively. Changes in temperature (from 26 to 40°C) had minor effects on TG (data not shown). Thrombin formation proceeds on plasma membranes and addition of exogenous phospholipids (4 μM, containing phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine at a ratio of 20:60:20) considerably enhanced kaolin-stimulated TG in mouse PPP (Figure 1B). TG was lower in whole blood as compared to PPP and addition of PL had no measurable effect on TG (Figure 1C). Plasma membranes of cells circulating in blood, e. g. platelets, provide the endogenous PL that promote FXIIa-driven TG (22). To assess the role of the hematocrit (HCT) in contact-initiated TG, mouse PPP was supplemented with increasing numbers of washed erythrocytes (Figure 1D). Increasing HCT levels

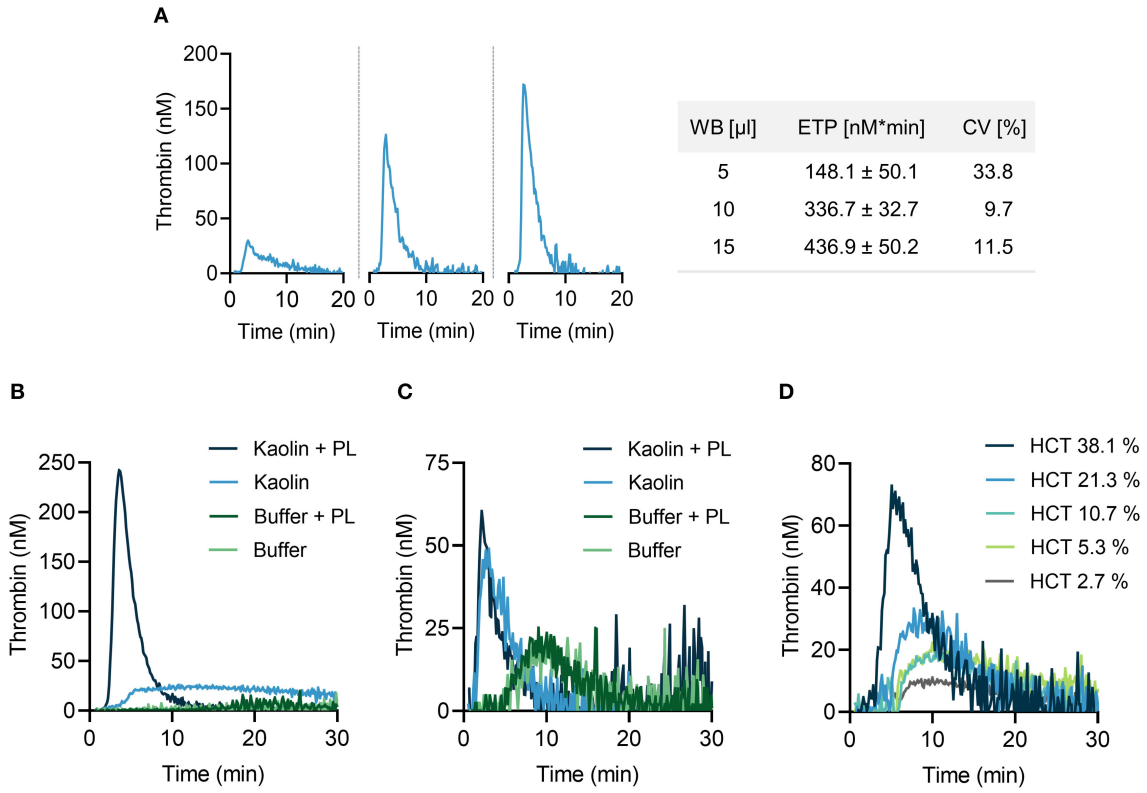

FIGURE 1
Thrombin generation (TG) in mouse whole blood assessed by CAT assay. **(A)** Left panel: real-time TG curves in 5 µl **(left)**, 10 µl **(middle)** or 15 µl **(right)** recalcified mouse citrate whole blood. Right panel: corresponding endogenous thrombin potential (ETP) values. Data are presented as mean ± standard deviation (SD) of 10 replicates and coefficient of variation (CV) was calculated thereof. **(B,C)** TG measurements performed in mouse PPP **(B)** and whole blood **(C)** in the presence or absence of phospholipids (PL, 4 µM). PPP and whole blood activated with buffer only served as control. **(D)** A fixed volume of mouse PPP was supplemented with increasing concentrations of erythrocytes as indicated by hematocrit levels (HCT, 2.7–38.1 %). In all experiments shown, TG was stimulated with kaolin (10 µg/ml). Representative TG curves measured in triplicates of $n = 3$ individual experiments.

dose-dependently increased kaolin-triggered TG, indicating a critical and unexpected procoagulant role of erythrocytes in TG (probably by their cell membranes).

For all further TG measurements, a reaction mixture containing 15% (v/v) mouse citrate-anticoagulated whole blood in 65 µl total reaction volume per well was used in the absence of exogenous PL at 33°C.

Activation of TG in mouse whole blood by various contact activators

TG in mouse whole blood was stimulated with different contact activators, including kaolin (Figures 2A–E), ellagic acid (Figures 2F–J) and synthetic long-chain polyP (≥400 mers; Figures 2K–O). All three contact activators dose-dependently increased total and maximum thrombin formation (peak thrombin), while reducing lag times and times to peak. Moreover, an array of commercially available contact activators from various manufacturers, commonly used in diagnostic coagulation laboratories to trigger aPTT assays, also effectively induced TG in mouse whole blood (Figures 3A–F). As synthetic polyP lacks calcium ions (Ca^{2+}) that are bound to the polymer under physiological conditions and largely alter the structure and function of the polymer (18, 23), we next tested natural polyP of cellular origin for its capacity to trigger TG. We purified polyP from confluent HEK293 cells by an established anion affinity chromatography-based method (17). Eluted material was resolved by urea/PAGE and visualized by DAPI-negative staining. The dye bound to polyanions such as nucleotides or glycosaminoglycans does not photobleach and appears white, whereas polyP bound to DAPI photobleaches rapidly resulting in a black stain. This procedure identified purified HEK293 cell-derived polyP with a chain length dispersity of ~400 to >1,000 mers, similar to the synthetic polyP P30 (Figure 4A), and allowed for assessing the amount of extracted polymers by densitometry analysis. Cell-derived polyP (7.1 and 10 µg/ml, respectively) potently triggered TG in mouse whole blood (Figure 4B), as indicated by a significantly shortened lag time compared

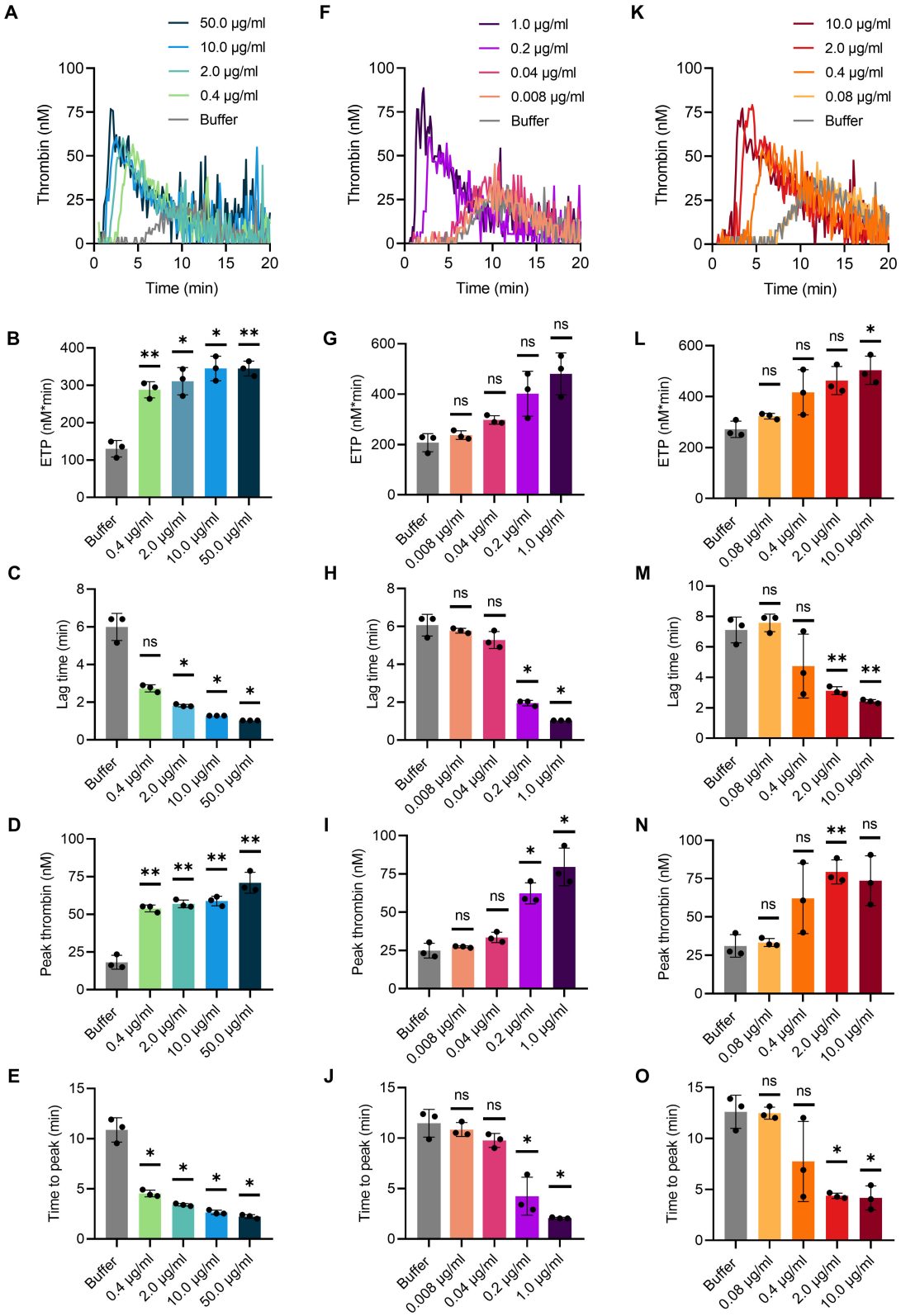

FIGURE 2
TG in mouse whole blood initiated by various contact activators. Citrate-anticoagulated venous whole blood collected from wild-type mice was supplemented in serial dilutions with **(A–E)** kaolin from 0.4 to 50.0 µg/ml, **(F–J)** ellagic acid from 0.008 to 1.0 µg/ml, and **(K–O)** synthetic polyP
(Continued)

FIGURE 2 (Continued)
(polymer size: ≥400 mers) from 0.08 to 10.0 μg/ml in buffer. **(A–C)** Representative real-time TG graphs of $n = 3$ independent experiments measured in triplicates each and calculated endogenous thrombin potential [ETP; **(B,G,L)**], lag times **(C,H,M)**, maximal thrombin [peak height; **(D,I,N)**] and time to maximal thrombin [time to peak; **(E,J,O)**] are shown. Each condition was measured in triplicates. Bars represent mean ± SD. One-way ANOVA followed by Dunnett's multiple comparisons test was performed to assess statistical significance. $P > 0.05$ (ns, not significant), $P < 0.05$ (*), $P < 0.01$ (**).

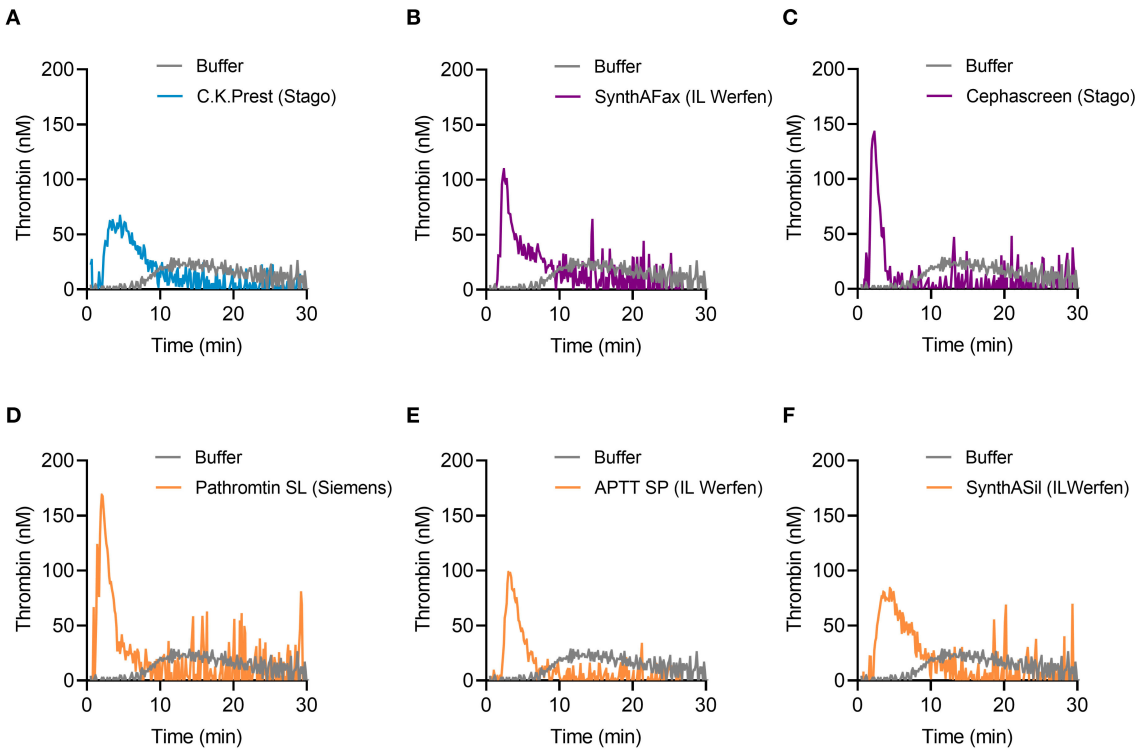

FIGURE 3
TG in mouse whole blood activated by commercially available aPTT reagents. **(A)** C.K.Prest (Stago), **(B)** SynthAFax (IL Werfen), **(C)** Cephascreen (Stago), **(D)** Pathromtin SL (Siemens), **(E)** APTT SP (IL Werfen), and **(F)** SynthASil (IL Werfen) were used with a final dilution of 1:20 each, to induce TG in mouse whole blood. Buffer serves as background control. Representative TG curves of $n = 3$ individual experiments are shown, while each condition was measured in triplicates.

to buffer control (Figure 4C). Additionally, we preincubated our cell-purified polyP with an exopolyphosphatase (PPX) variant that lacks the domains 1 and 2 (PPX_Δ12) and binds specifically to polyP (but not to other polyanions) without degrading the polymers (21). Preincubation of anion exchange-purified polyP with PPX_Δ12 completely blocked polyP-induced TG, leading to lag times indistinguishable to buffer control, confirming that TG is exclusively driven by polyP.

FXII dependency of contact activator-stimulated TG in mouse blood

To confirm that TG is triggered by different negatively charged surfaces, we performed the CAT assay in whole blood of $F12^{-/-}$ mice. Previously we have shown that FXII is not detectable in plasma of $F12^{-/-}$mice by Western blotting (14). Kaolin (Figure 5A), ellagic acid (Figure 5B) and synthetic polyP (Figure 5C) completely failed to induce TG in $F12^{-/-}$ mouse whole blood. Reconstitution of blood samples from $F12^{-/-}$ mice with human plasma-derived FXII at physiological concentration (375 nM) fully restored defective TG upon stimulation with contact activators. Furthermore, we analyzed kaolin-induced TG in relation to FXII levels below and above the physiological concentration (Figure 5D). Supraphysiological FXII levels (750 nM) shortened the lag time, whereas reduced FXII concentrations (0–187.5 nM) prolonged lag times. The prolongation reached significant values at FXII <1.5 nM (Figure 5E). As pharmacological targeting of the FXII substrate FXI is gaining interest for safe interference with thromboembolism, we analyzed effects of altered FXI

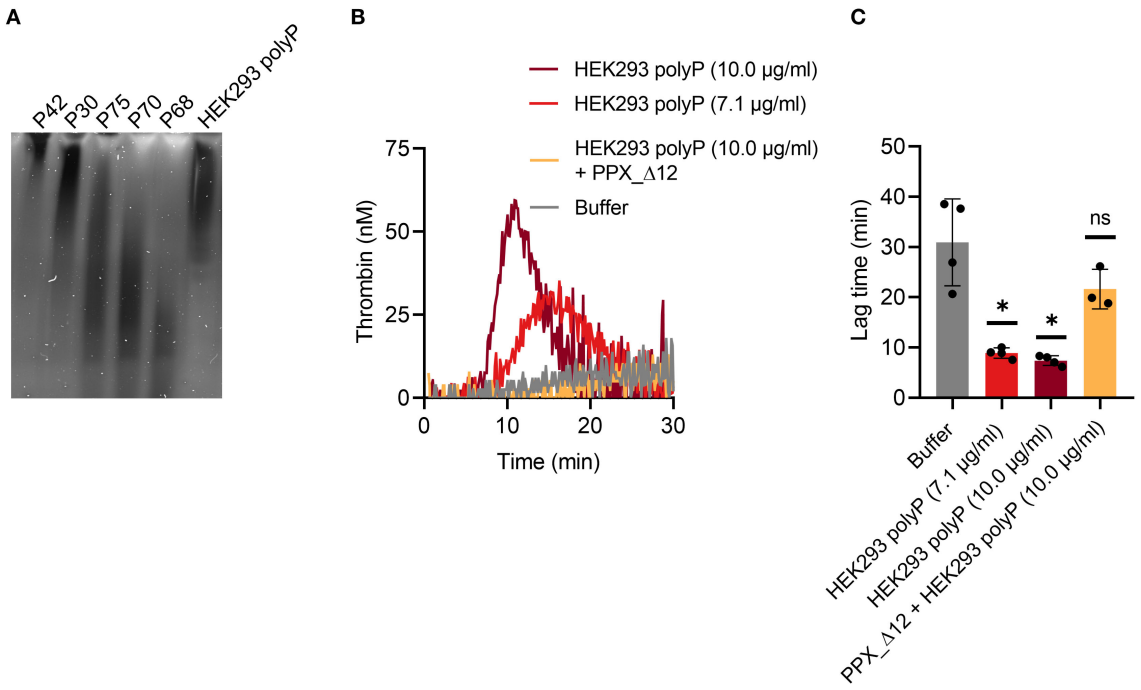

FIGURE 4
Cell-derived polyP triggers TG in mouse whole blood. **(A)** PolyP was isolated from 1.1 × 10^6 HEK293 cells by anion exchange chromatography. Purified polyanions (50 ng/lane) were resolved by denaturating urea-PAGE [7 M urea/15% (w/v) polyacrylamide gels] and visualized using negative DAPI-staining. 20 ng/lane of synthetic polyP (P42–P68) was loaded as size and mass standard. Chain length of synthetic polyP - P 42: ≥ 650 mers, P30: ≥ 400 mers, P75: ≤ 650 mers, P70: ≤ 400 mers, P68: ≤ 150 mers. **(B)** Real-time TG in mouse whole blood was stimulated with cell-purified polyP (7.1 and 10 µg/ml, respectively) in presence or absence of recombinant polyP inhibitor PPX_Δ12 (500 µg/ml). Representative TG curves of $n = 3$ individual experiments measured in triplicates each. Corresponding time that elapses until TG is initiated (lag time) in **(B)** is shown in **(C)**. Mean ± SD are indicated in bars. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. PolyP-stimulated TG was compared to buffer. $P > 0.05$ (ns, not significant), $P < 0.05$ (*).

concentrations on TG in mouse whole blood. Lowering FXI levels dose-dependently reduced contact activation-triggered TG (Figure 5F) and prolonged lag times upon kaolin stimulation (Figure 5G).

Consistent with impaired TG in FXII-deficient whole blood, addition of the specific FXIIa inhibitor rHA-infestin-4 [Inf-4, 500 µg/ml (24)] to blood of WT mice completely abolished TG induced by kaolin (Figure 6A), ellagic acid (Figure 6B) and polyP (Figure 6C). FXIIa is produced by zymogen binding to negatively charged surfaces or proteolytic conversion of FXII by PKa. Importantly, blocking PKa by aprotinin (200 KIU/ml) reduced kaolin, ellagic acid and polyP-triggered TG (Figures 6A–C). These data are consistent with the notion that TG in mouse whole blood is critically dependent on FXII contact activation, while contact activation-formed FXIIa is amplified by PKa-mediated FXII fluid phase activation. To evaluate our assay for analysis of currently used anticoagulants, we spiked mouse whole blood with DOACs. The presence of the factor Xa inhibitors apixaban or edoxaban blocked contact activation-triggered TG in a dose-dependent manner (Figures 6D,E). In a proof-of-concept experiment, the direct thrombin inhibitor dabigatran completely abolished kaolin-induced TG at a dose of 100 ng/ml (Figure 6F).

Discussion

Thromboembolic and bleeding diseases represent a significant medical burden. Coagulation diagnostics are required to assess risk of thrombosis or bleeding, monitor anticoagulation or substitution therapy, and provide a standard of care for patients with coagulation abnormalities. Coagulation diagnostics include analysis of plasma (e.g., activity of factor VIII), platelets (e.g., ADP release) and recently whole blood. The latter allows to assess plasma coagulation, procoagulant activity of circulating cells including platelets and leukocytes, and the intimate crosstalk thereof. In addition to platelets, the presence or absence of leukocytes such as monocytes and neutrophils plays a major role in secondary haemostasis, e.g., by exposing tissue factor (TF) (25), initiating the extrinsic coagulation pathway. Activated eosinophils, macrophages, and

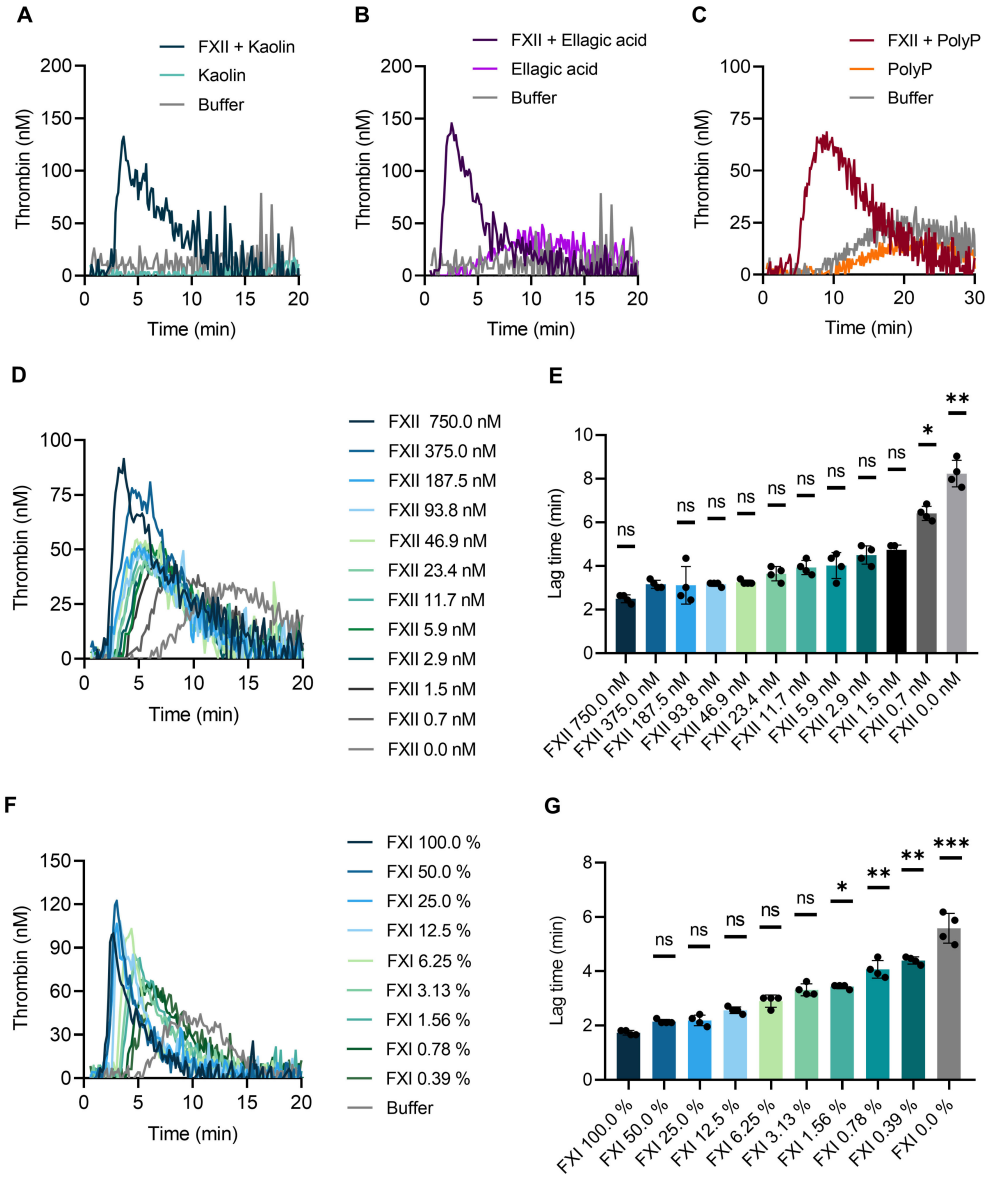

FIGURE 5
Contact-triggered TG is dependent on FXII and FXI levels in mouse whole blood. Venous whole blood collected from FXII-deficient (*F12*$^{-/-}$) mice was stimulated with **(A)** kaolin (10 μg/ml), **(B)** ellagic acid (1 μg/ml), and **(C)** synthetic long-chain polyP (10 μg/ml, polymer size: ≥400 mers). **(A–C)** Real-time TG was assessed in recalcified blood following addition of human FXII (375 nM) or buffer. **(D,E)** Plasma-derived human FXII was added to whole blood collected from *F12*$^{-/-}$ mice at concentrations ranging from 0 to 750 nM. **(F,G)** Defined mixtures of whole blood from wild-type and FXI-deficient (*F11*$^{-/-}$) mice resulted in FXI levels of 100–0%. **(D–G)** Kaolin (10 μg/ml) stimulated TG. Corresponding times to onset of TG (lag times) in **(D,F)** are shown in **(E,G)**. Bars represent mean ± SD. Statistical significance was assessed by performing the Kruskal-Wallis test followed by Dunn's multiple comparison, with lag times compared to **(E)** 375 nM FXII or **(G)** 100% FXI. $P > 0.05$ (ns, not significant), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

most especially neutrophils release anionic extracellular DNA into the extracellular space (neutrophil extracellular traps, NETs), resulting in FXII activation and degradation of tissue factor pathway inhibitor (TFPI) (26–28).

CAT-based TG in human PPP and PRP is a sensitive method for monitoring formation and neutralization of the main executor of coagulation, thrombin. Tchaikovski et al. adapted the human CAT assay for mouse PPP and showed that mouse plasma appears to have higher procoagulant activity as compared to human samples, characterized by very short lag times (29). In fact, monitoring TG from its initial phase and calculating TG parameters was challenging. To overcome this limitation, the authors analyzed TG at low temperature of 33°C, which caused the procoagulant

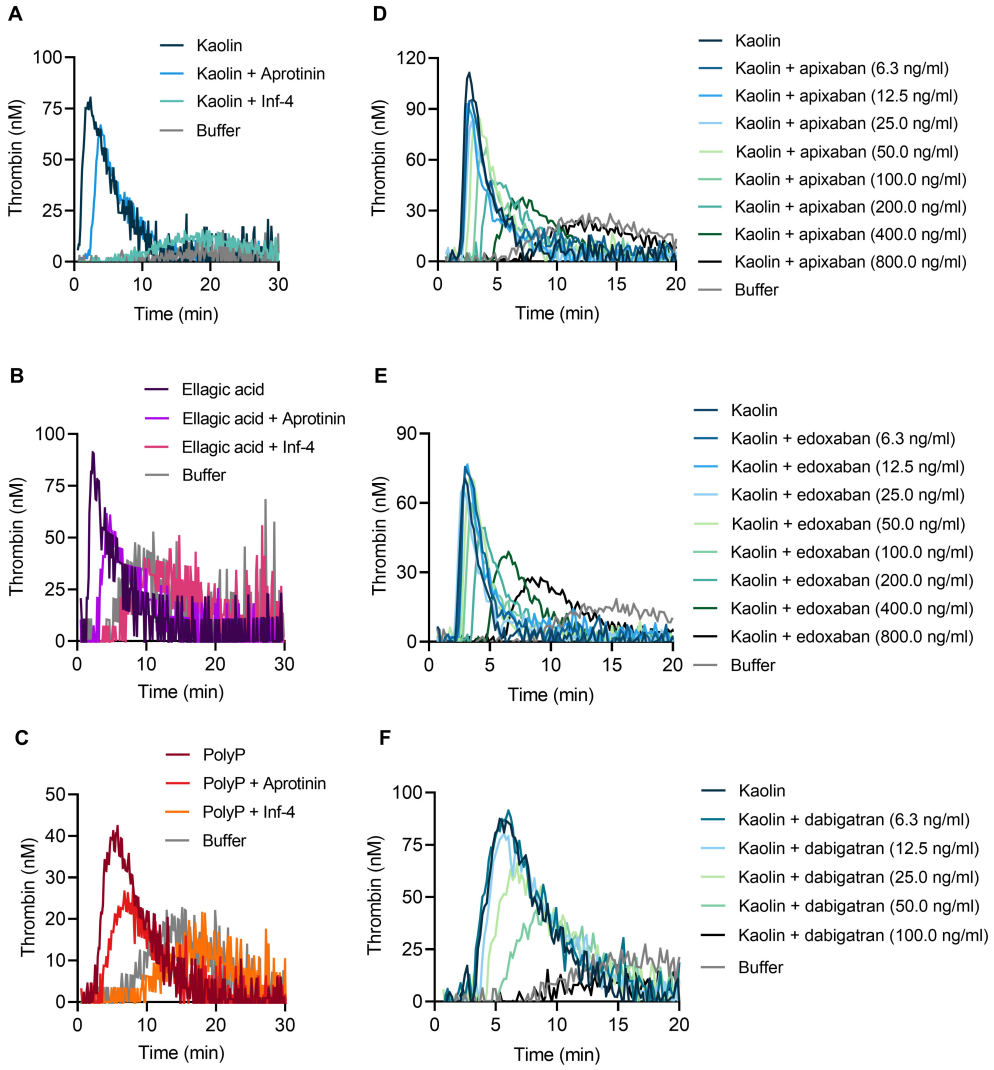

FIGURE 6
Targeting FXIIa, PKa, factor Xa, and thrombin reduces contact-triggered TG in murine whole blood. Representative thrombograms of citrate-anticoagulated whole blood obtained from WT mice stimulated with **(A,D–F)** kaolin (10 μg/ml), **(B)** ellagic acid (1 μg/ml), and **(C)** synthetic long-chain polyP (10 μg/ml, polymer size: ≥400 mers) in the presence or absence of **(A–C)** rHA-infestin-4 (Inf-4, 500 μg/ml) and aprotinin (200 KIU/ml) or direct oral anticoagulants (DOACs): **(D)** apixaban, **(E)** edoxaban, and **(F)** dabigatran. DOACs were added in serial dilutions from **(D,E)** 6.3 to 800 ng/ml or **(F)** 6.3 to 100 ng/ml. Buffer serves as background control. Representative curves of three individual experiments are shown. Each condition was measured in triplicates.

reactions to slow down and prolonged lag times. We adapted this strategy for our study, noting that performing our CAT assay at different temperatures had only minor effects on TG profiles.

Monitoring TG in human and mouse whole blood required modifications of existing protocols as erythrocytes rapidly sediment, quench fluorescence signals and contribute to clot contraction during TG measurements. Whole blood TG assays are further limited by high intra-assay variation (4), handling issues (5) or the incapacity to analyze multiple samples simultaneously (6, 7). Recently, a fluorogenic TG assay in human whole blood with constant sample mixing by permanently moving the microtiter plate led to highly reproducible data (8). Furthermore, established TG analysis of mouse whole blood used filter paper disks to minimize erythrocyte sedimentation by restraining them in a matrix (10). However, filter papers function as contact activators leading to significant preanalytical FXIIa formation, limiting the analysis of the intrinsic pathway of coagulation. Here, we developed a novel assay that allows to reliably measure TG triggered by contact activation in mouse whole blood in the absence of filter papers, while data variability between replicates (Figure 1) was comparable to the filter paper-

based TG technique presented by Ninivaggi and colleagues (10). In contrast to previous TG assays (29), we used about half of the blood sample volume allowing for multiple analyses from minimal blood volumes.

To stimulate TG in mouse whole blood in a dose-dependent manner in our assay (Figure 2), we used the polyanions kaolin, ellagic acid and synthetic polyP (Figure 2), which are known to activate FXII (30–32). Of note, the chain length of synthetic polyP correlates with its procoagulant potential in plasma *ex vivo* (32), whereas natural polyP is complexed with Ca^{2+} and forms insoluble nanoparticles challenging a role for polyP chain length for coagulation *in vivo* (23). Ca^{2+}-polyP microparticles induce FXII activation on procoagulant platelet surfaces (18, 23), various cancer cells, and extracellular tumor-derived vesicles such as prostasomes, and drive pulmonary embolism in mouse models (16, 17). Because of this critical role in triggering thromboembolic events in animals, we also tested the potential of natural polyP isolated from HEK293 cells in stimulating TG in mouse whole blood. Methods for isolation of polyP from cells and cell-derived components are either based on phenol/chloroform extraction (33) or anion exchange chromatography (34, 35). While the former method extracts predominantly water-soluble (short-chain) polyP, preparation by anion exchange resin contains both soluble (short-chain) and insoluble (long-chain) polyP. Indeed, we isolated long-chain polyP (~400 to >1000 mers) from confluent HEK293 cells using commercially available ion exchange resins (Figure 4). Similarly, synthetic polyP (Figure 2), cell-derived polyP triggered TG in mouse whole blood (Figure 4). PolyP extracted with anion exchangers used for DNA purification has been claimed to be contaminated with procoagulant silica that presumably had leaked from the column matrix (36). However, targeting cell-derived polyP with recombinant PPX_Δ12 that binds specifically to polyP of chain length >35 but not to other polyanions (21), completely inhibited TG. These data indicate that silica, if present at all, has minor importance in TG induced by anion exchanger-purified polyP using our method.

In addition to polyanions, extracellular vesicles (EV) derived from platelets and erythrocytes have the potential to stimulate TG (37). Erythrocyte-derived EV contribute to intrinsic pathway-mediated TG by two mechanisms: (i) contact activation of FXII and (ii) a FXII-independent pathway driven via PK activation and subsequent stimulation of factor IX (FIX) (38). Consistent with this study, PKa may activate FIX *in vitro* (39). FXII deficiency or blocking FXIIa by Inf-4, but not PK inhibition with aprotinin (Figure 4), completely abrogated TG in mouse whole blood exposed to polyanionic surfaces. These data support the notion that TG was initiated through classical FXII contact activation, but not via a FXII-independent mechanism. The discrepancy may reflect the fact that FXII-independent procoagulant mechanisms result from long-term storage of erythrocytes (38).

Our novel CAT assay requires further analysis, for example, in animal models with subtle and diffuse coagulation disturbances, including hypofibrinolysis in acute sepsis and traumatic injury. In severe sepsis, widespread activation of the coagulation system involves rapid "consumption" of several clotting factors with persistent thrombus formation. This leads to a hypocoagulable state (40) termed disseminated intravascular coagulation (DIC) that is associated with multiorgan failure and high mortality. Sepsis-mediated DIC is characterized by excessive formation of thrombin in which pathogen-derived polyP activates FXII (41). Clinically, DIC resembles trauma-induced coagulopathy (TIC), where patients initially tend to bleed but later change to a more hypercoagulable state associated with venous thromboembolism and multiorgan failure (42). Our CAT assay in mouse whole blood could embed TG in a more physiologic setting and offer additional insight into these complex coagulopathies.

In conclusion, we established a method to specifically assess TG, driven via the plasma contact system in mouse whole blood. Since different tested polyanions triggered FXIIa-mediated TG, our method provides a useful tool to identify other, yet unknown candidates contributing to TG through contact activation. As FXII deficiency or FXIIa inhibition led to defective TG in mouse whole blood, our technique may be of interest for the development of antithrombotic drugs, as FXII inhibitors interfere with thrombosis while sparing hemostasis (43, 44).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

SK performed the experiments and analyzed the data of this study. RM, HE, MB, MF, PK, RP, CM, LB, MR, and BL provided critical tools, discussed data, and critically read the manuscript. SK and TR wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors BL and MR are employees of Synapse Research Institute, part of Diagnostica Stago.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1008410/full#supplementary-material

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Application of the thrombin generation assay in patients with antiphospholipid syndrome: A systematic review of the literature

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Background: The antiphospholipid syndrome (APS) is classified by the presence of antiphospholipid antibodies (aPL) and thrombotic and/or adverse obstetric outcomes. The diagnosis and risk assessment of APS is challenging. This systematic review investigated if the thrombin generation (TG) assay could be helpful for APS diagnosis and risk assessment.
Methods: A systemic review was performed by searching two databases (MEDLINE and Embase) until March 31, 2022, using a search strategy with two concepts: APS and TG, and related keywords. Two reviewers independently screened the articles based on predefined inclusion and exclusion criteria. Data extraction and quality assessment with the Newcastle-Ottawa Scale (NOS) were performed independently. Synthesis Without Meta-analysis guidelines were followed for data synthesis reporting.
Results: Fourteen studies with 677 APS and 1,349 control subjects were included with variable quality according to the NOS. Twelve studies measured TG *via* the calibrated automated thrombogram (CAT) method using a fluorogenic substrate, whereas two used a chromogenic substrate-based TG assay. One study compared the CAT assay to the fully-automated ST Genesia® (Stago, France). Two studies initiated TG using platelet-rich plasma, whereas the rest of the studies used platelet-poor plasma. Resistance to activated protein C (aPC) was examined in ten studies. They reported a significant increase in aPC-resistance in APS patients compared to healthy controls, aPL-carriers, and thrombotic controls. Based on two studies, the prevalence of aPC-resistance was higher in APS patients compared to healthy controls and thrombotic controls with odds ratios of 5.9 and 6.8–12.8, respectively ($p < 0.05$). In contrast, no significant difference in aPC-resistance was found between APS patients and autoimmune disease controls. Furthermore, 7/14 studies reported TG-parameters including peak height, endogenous thrombin potential, lag time, and time to peak, but these outcomes were highly variable between studies. Furthermore, TG methodology between studies differed greatly, impacting the comparability of the studies.
Conclusion: aPC-resistance measured with TG was increased in APS patients compared to healthy and thrombotic controls, but the diagnostic and prognostic value is unclear compared to current diagnostic strategies. Studies of other TG-parameters were heterogeneous and more research is needed to identify their potential added value in APS diagnosis.
Systematic Review Registration: https://www.PROSPERO/, identifier: CRD42022308363

KEYWORDS

anitphospholipid antibodies, antiphospholid syndrome, pregnancy outcome, thrombin generation, thrombosis

Introduction

The antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterised by the persistent presence of antiphospholipid antibodies (aPL) and clinical features of thrombosis and/or pregnancy morbidity (1). aPL are a heterogeneous population of circulating immunoglobulins including anticardiolipin antibodies (aCL), anti-β2-glycoprotein I antibodies (aβ2GPI), and lupus anticoagulant (LA) (1). The clinical phenotype associated with APS is highly heterogeneous, and pathophysiological mechanisms are still not fully understood. Despite the progress in unravelling the pathogenesis of APS (2), difficulties remain in identifying patients at high thrombotic and obstetric risk.

The diagnosis of APS relies predominantly on laboratory testing of aPL due to the relatively high incidence of clinical manifestations such as thrombosis and obstetric complications in the general population. Therefore, the laboratory detection of aPL is required to confirm the clinical suspicion of APS. The classic tests for the detection of aPL comprise clotting-based assays for the detection of LA and solid phase immunoassays for the detection of aCL and aβ2GPI IgG/IgM antibodies (3). Although various studies have established an association between these laboratory assays and the clinical manifestations of APS (4–7), none of these assays is considered as the gold standard in APS. Consequently, several other laboratory tests and diagnostic tools are being investigated that may help to improve the diagnosis and risk evaluation in patients with APS.

Recently, interests have focused on more global coagulation assays such as the thrombin generation (TG) assay. The TG assay measures both thrombin formation and inhibition, which represents a significant part of the overall coagulation process. Because of this, TG reflects more closely what occurs *in vivo* compared to clotting-based assays by measuring the dynamic processes of thrombin generation (8–10). Furthermore, TG tests can be used to study the contribution of procoagulants and anticoagulants in patients with various haemostatic disorders (11, 12), or to investigate the impact of medication on haemostasis (13, 14). TG methods lack standardisation (15), but while efforts are ongoing to improve this issue, the TG assay appears to be a valuable tool for determining the increased tendency to form blood clots (hypercoagulability) in patients with a wide variety of thrombotic disorders (11, 14). In addition, TG assays can also be used to assess activated protein C (aPC) resistance (aPC-r), a possible contributor to hypercoagulability in APS patients (16).

This systematic review aimed to investigate if the TG assay could be used as a diagnostic tool for, on one hand, the diagnosis of patients with APS and, on the other hand, for identifying APS patients at high risk for recurrent clinical manifestations.

Methods

Protocol and registration

This systematic review was registered in the International Prospective Register of Systematic Reviews (PROSPERO), registration number CRD42022308363. The review was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) 2020 guidelines (17).

Search strategy

Two databases (MEDLINE *via* the PubMed interface and Embase *via* the Embase.com interface) were searched for the combination of two concepts (APS and TG) according to the search strategy specified in **Supplementary Figure S1**. No restrictions such as language or date specification were applied. Validation of the search strategy was performed by adequate identification of a validation set of six studies specified by K.D. (**Supplementary Table S1**). The search was performed on November 3, 2021. An e-mail alert was activated on both databases to receive weekly updates and these studies were included for evaluation until March 31, 2022.

Eligibility and selection process

Duplicate removal was performed in EndNote 20 (Clarivate, London, United Kingdom) with manual verification. Deduplicated records were transferred to Rayyan (Rayyan Systems Inc., Cambridge, MA, United States) for the screening process (18). Two researchers (R.G. and A.V.) independently reviewed all records for eligibility based on title and abstract. After the screening stage, the same reviewers independently evaluated the full texts of the included articles. In case of disagreement, a third reviewer (K.D.) was consulted to reach a consensus at both evaluation stages. Eligibility was checked against predefined criteria (**Table 1**). Percentage agreement and Cohen's kappa values were determined at both stages.

Data extraction and quality assessment

Data extraction and quality assessment were performed independently by two reviewers (R.G. and A.V.). Extracted data were registered in a table agreed upon beforehand in Excel (Microsoft, Redmond, WA, United States.). Data items that were extracted are summarised in **Supplementary Table S2**. A third reviewer (K.D.) was consulted in case of disagreement when discussion between the two reviewers did not lead to consensus. The Newcastle-Ottawa Scale (NOS) was applied to assess the quality of the included cohort and case-control studies (19). An adapted NOS was used for scoring studies with a cross-sectional design (**Supplementary Figure S2**) (20). In the (adapted) NOS, each study was attributed stars for three main items (selection, comparability, and outcome/exposure). The number of stars was added up per study, resulting in a score of maximally nine stars for case-control studies and seven stars for cross-sectional studies. The higher the score, the higher the expected quality and the lower the risk for bias in the study. High-quality case-control studies are mostly considered with a NOS score of seven or

TABLE 1 Eligibility criteria for assessing inclusion and exclusion of retrieved research.

Variable	Inclusion	Exclusion
Population	– Adult patients diagnosed with the antiphospholipid syndrome or who met the Sapporo/Sydney classification criteria.	– Underaged patients (<18 years). – No exclusion based on sample size.
Exposure	– Assessment of thrombin generation with a thrombin generation assay.	– No exclusion based on type of thrombin generation assay used.
Control	– Laboratory assays: antiphospholipid antibodies included in classification criteria. – Clinical: patients without antiphospholipid syndrome.	– Studies describing only laboratory parameters not included in classification criteria (e.g. anti-phosphatidylserine/prothrombin antibodies).
Outcomes	– Description of thrombin generation in relation to classification of antiphospholipid syndrome or related clinical risk (thrombosis or pregnancy morbidity).	– Studies describing only clinical manifestations not included in classification criteria (e.g. thrombocytopenia). – No thrombin generation results or derived parameters reported.
Study design	– Observational studies including case-control, cohort, and cross-sectional studies.	– Article type: letter, conference abstract, (systematic) review, editorials. – Non-human studies. – Experiments based on patient-derived material such as isolated antibodies. – Language other than English.

more, while no generally accepted threshold is available for cross-sectional studies. Both reviewers independently provided a score for each included article, and it was reported after a consensus was reached between the two reviewers.

Data synthesis

No meta-analysis was performed owing to the incomplete reporting of effect estimates and diversity of exposures (TG methodologies), control groups and study designs. Synthesis Without Meta-analysis (SWiM) guidelines were followed for data synthesis reporting (21). Studies were grouped based on the outcome reported, being aPC-r and other TG-derived parameters, because of their different underlying concept. Studies may however report both outcomes and can therefore be included in both groups. No standardised metric could be used to describe continuous data from aPC-r results because of the variety in TG methods used to assess aPC-r. It was not possible to combine *p*-values because exact values were not always available, but the direction of the effect was synthesised. Unadjusted odds ratios (ORs) were calculated without pooling if a clinical cut-off value was available. Data were presented per group in a tabular format (separately for continuous and dichotomous data) and ungrouped graphically in a modified effect direction plot with studies ordered according to the score received during the quality assessment, followed by study patient group size and split by study design (22). No formal heterogeneity assessment was performed.

Results

Publication characteristics

In total, 1,116 records were retrieved from the two databases. After removing duplicates, 756 records were screened for eligibility, of which 26 were selected for full-text evaluation. After full-text evaluation, 14 records were included for data extraction (**Figure 1**). The agreement between the two reviewers for the abstract and full-text screening was substantial with values of 99% (Cohen's κ: 0.72) and 81% (Cohen's κ: 0.61), respectively. Reasons for exclusion of the articles that were subject to full-text evaluation can be found in **Supplementary Table S3** (23–34). Only two of the 14 included studies were performed outside of Europe (one in Japan and one in Canada). We identified multiple records from the same research groups. We could rule out overlapping populations between studies for some research groups, although for three studies overlap is not unlikely since they use patient populations recruited under the same ethical reference number (35–37). However, the information provided in these three studies is relevant and unique since they compare the APS patient population against distinct control groups. All studies were retrospective and had an observational design, of which four were identified as cross-sectional studies, ten case-control studies, and zero cohort studies. Characteristics and main findings of all included studies are summarised in **Supplementary Table S4** (35–48). Only one record described information on the ethnicity of the cases (37). Therefore, information on ethnic characteristics was not further reported.

Quality assessment

The quality assessment based on the NOS is shown for each study in **Supplementary Table S5**. Only one study reached a NOS score of 7 and could be considered of high quality. All other case-control studies had a score of 5–6 except for one study that scored 3 stars. Three cross-sectional studies had a score of 4, and one study scored 3 stars. One study received points in the comparability category since no other studies controlled for confounders such as age or gender.

Patient and control characteristics

Six hundred and seventy-seven APS cases were reported. APS can be classified as thrombotic and obstetric APS (1). Most studies identified thrombotic and obstetric manifestations retrospectively based on medical records. However, only eight studies specified the location or type of thrombotic and obstetric adverse events (35, 37, 38, 43, 45, 46, 36, 44). Two studies

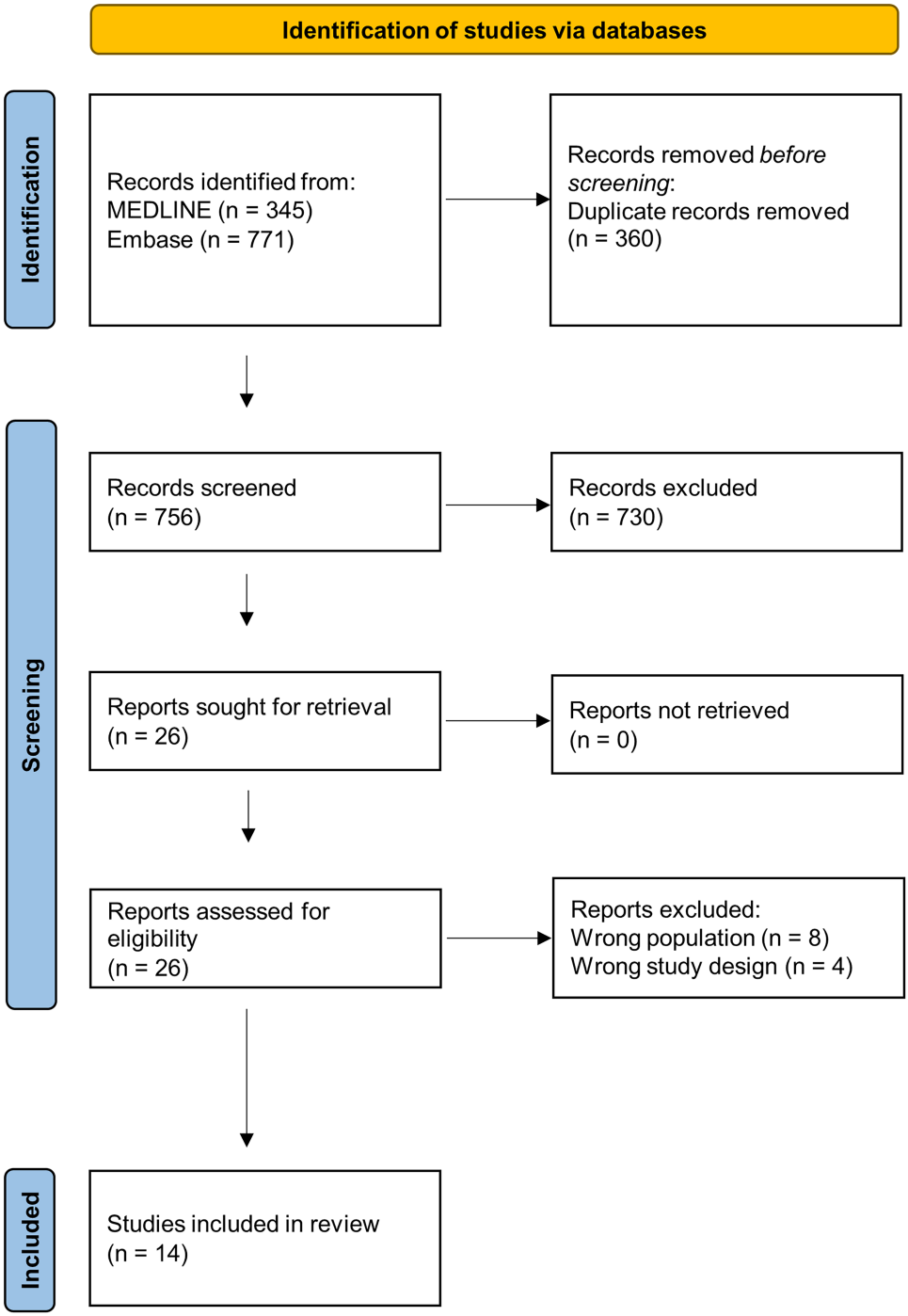

FIGURE 1
Flow chart of the systematic search of the literature and study selection process.

distinguished thrombotic and obstetric APS patients as separate patient groups (36, 38), including 118 thrombotic APS patients and 34 obstetric APS patients (36, 38). Four studies included thrombotic APS patients ($n = 102$) only (35, 37, 43, 47). Four studies specified the proportion of APS patients with thrombotic manifestations ($n = 200$), obstetric manifestations ($n = 37$), or both ($n = 14$) but did not analyse the subgroups separately (40, 41, 45, 46). In four studies, no clear differentiation between thrombotic and obstetric APS patients could be derived from the publications ($n = 172$) (39, 42, 44, 48). In addition, APS patients can be classified according to the presence (i.e., secondary APS) or absence (i.e., primary APS) of any coexisting autoimmune disease (AID) (49). In one study, only secondary APS patients ($n = 24$) were included (37). Three studies specified whether patients had primary ($n = 168$) or secondary ($n = 39$) APS and interpreted the subgroups separately (36, 44, 48). The presence of

AID was not specified in 212 APS patients (39, 41–43, 46, 47). Furthermore, in the remaining studies 42 secondary APS and 192 primary APS patients were investigated, but no subgroup analysis was performed in these studies (35, 38, 40, 45).

In total, 1,349 control cases were reported. Control cases can be categorised into four groups according to the clinical presentation reported. Group 1 included 127 cases that were persistently positive for aPL but did not fulfil the clinical criteria manifestations of APS. Group 2 included 207 thrombotic control patients with a history of thromboembolic complications but did not fulfil the APS laboratory criteria (3). Group 3 included 214 AID patients with conditions other than APS. Most of these patients had systemic lupus erythematosus (SLE) ($n = 179$), either isolated SLE (67/179), with the presence of aPL (69/179), or with a history of thrombotic complications (14/179), but none of these patients fulfilled the APS criteria (3). Group 4 included 639 presumably healthy control cases. Three studies included patient groups that could not be described by the groups above (40, 45, 46). De Laat-Kremers et al. included 93 hospital controls, which were patients visiting the hospital for conditions other than APS (40). In addition, two studies reported anticoagulant-matched controls that did not meet the APS classification criteria (45, 46). Kremers et al. included 31 vitamin K antagonist (VKA) treated control cases treated for indications other than APS (45). Liestøl et al. included 38 patients on long-term warfarin therapy for non-valvular atrial fibrillation (46).

Thrombin generation methodology

Preanalytical and analytical aspects of all included studies are summarised in **Table 2**. Twelve articles measured TG *via* the calibrated automated thrombogram (CAT) method, according to the principles described by Hemker et al. in 2003. One study compared the CAT assay to the ST Genesia® (Stago, Asnières-sur-Seine, France) methodology, an automated TG analyser based on the same principles as the CAT assay (36). TG methods can be performed using platelet-rich plasma (PRP), platelet-poor plasma (PPP), or whole blood. Zuily et al. and Foret et al. initiated TG in PRP (42, 48), whereas the rest of the studies used PPP. The platelets in the PRP provide a natural source of phospholipids. However, in PPP, negatively charged phospholipids need to be added to the reaction for optimal TG. In most publications, the use of synthetic phospholipids at a final concentration of 4.0 μM was reported (35–38, 40, 45–47), although only two studies specified the phospholipid composition (45, 46). Devreese et al. lowered the phospholipid concentration to 1.0 μM to raise the phospholipid dependency of the assay (41). Bloemen et al. used inverted erythrocyte membranes as a phospholipid surface (39). In all studies with CAT, TG was triggered by tissue factor (TF). The final TF concentration was 5.0 pM for the majority of the studies using the CAT method (35–37, 39–41, 45, 46). Two studies also included a lower TF concentration of 1.0 pM to make the TG measurement more sensitive to the intrinsic coagulation pathway (40, 45). Billoir et al. and Zuily et al. did not use 5.0 pM TF to trigger TG but used a lower TF concentration of 1.0 pM and 0.5 pM, respectively (38, 48). In addition, Matsumoto et al. initiated TG utilising a mixture of TF (0.5 pM) and ellagic acid (0.3 μM) to investigate the thrombogram but used 1.0 pM TF to assess aPC-r. This reaction mixture can trigger thrombin generation *via* both intrinsic and extrinsic coagulation mechanisms (47). Finally, Foret et al. reported the use of a low TF concentration to initiate TG, but no exact TF concentration was reported (42).

Furthermore, Green et al. and Hanly et al. reported a TG method different from CAT, using a chromogenic substrate for thrombin instead of a fluorogenic substrate used in CAT (43, 44). Green et al. used thromboplastin (Recombiplastin 2 G, Werfen, Spain) as TF source with a final concentration of 1.5 pM TF and 10 μg/ml unspecified phospholipids to trigger thrombin generation in PPP (43). Hanly et al. also used thromboplastin (Sigma, St. Louis, MO, United States), in a final dilution of 1/80 as trigger reagent (44).

Resistance to aPC may be assessed by measuring and comparing TG with and without adding a protein C pathway activator (or aPC itself) to the sample. aPC-r was assessed in 71% (10/14) of all included studies (35–38, 42, 43, 45–48). In these studies, aPC-r was determined by adding aPC (5/10 studies) (38, 42, 46–48), or a protein C activator isolated from snake venom, Protac® (Pentapharm AG, Switzerland) (1/10 studies) (43) to a TG assay. Two studies used both Protac® and aPC to determine aPC-r (35, 37). One study used recombinant human thrombomodulin (TM) (Asahi Kasei Pharma, Beijing, China) to mediate protein C activation (45). In another study, aPC-r was determined using the CAT TG method by adding aPC and Protac® and using the TG analyser ST-Genesia® that uses TM from purified rabbit lung (36).

Anticoagulants can influence TG results. The use of anticoagulation therapy was reported in eight out of the 14 articles (35–37, 40–42, 45, 46). Devreese et al. and Liestøl et al. equally included patients with and without oral anticoagulant therapy. They reported that 18% (INR: 0.97–2.44) and 65% (INR: 1.6–4.3) of the total study population were on VKA treatment, respectively (41, 46). In both studies patient plasma was mixed with equal volumes of pooled normal plasma (PNP) to correct for reduced coagulation factor activity as a result of VKA treatment (41, 46). Ramirez et al. specified that 79.2% of the included APS patients were on anticoagulant therapy at the time of blood sampling (37). Of these patients, 1/19 was on low molecular weight heparin (LMWH), 4/19 on direct oral anticoagulants (DOACs), and 14/19 on VKA. To neutralise the effects of this anticoagulant therapy they reported mixing of patient plasma with equal volumes of PNP (37). In addition, Kremers et al. investigated the effect of VKA treatment on TG by including VKA-treated APS patients ($n = 50$), APS patients without VKA treatments ($n = 30$), and age- and gender-matched VKA-treated control subjects ($n = 31$) (45). De Laat-Kremers et al. described a validation cohort of APS patients and specified that 82% of these patients were on anticoagulants without describing further details (40). Foret et al. reported that 1.7% of the total population was on LMWH, whereas 45.3% was on VKA treatment (42). However, these percentages also include control patients, and details regarding

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TABLE 2 Preanalytical and analytical aspects of the included studies.

First author	Year	Preanalytical					Thrombin generation methodology									
		PPP/ PRP	Sample preparation	Storage	Thawing	Mixing with PNP	CAT	Instrument	Software	Replicate amount	TF f.c.	TF origin	PL f.c.	PL origin	Nor-malised	Normalisation method
Arachchillage	2014	PPP	Citrated plasma, double centrifugation: 15 min., 2000 *g* at RT	−80°C	10 min. 37 °C WWB	Yes 1:1 (sample: PNP)	Yes	n.s.	n.s.	Triplicate	5.0 pM	Synthetic	4.0 µM	Synthetic	Yes	Against in-house prepared PNP in each run
Billoir	2021	PPP	n.s.	n.s.	n.s.	No	n.s.	Fluoroskan Ascent (Thermo Fisher Scientific, Finland)	n.s.	n.s.	1.0 pM	Synthetic	4.0 µM	Synthetic	No	Not applicable
Bloemen	2016	PPP	Citrated plasma, filtration (0.2 µm filter)	n.s.	n.s.	No	Yes	n.s.	Thrombinoscope (Netherlands), version n.s.	n.s.	5.0 pM	Recom-binant, human (Dade Behring, Germany)	n.s.	Erythrocyte membranes	No	Not applicable
De Laat – Kremers	2021	PPP	Citrated plasma, double centrifugation: 10 min., 2821*g*	n.s.	n.s.	No	Yes	n.s.	n.s.	n.s.	1.0 pM 5.0 pM	Synthetic	4.0 µM	Synthetic	No	Not applicable
Devreese	2010	PPP	Citrated plasma, double centrifugation: 15 min., 2000*g* and 10 min.,>2500 *g* at RT	−80°C	n.s.	Yes 1:1 (sample: PNP)	Yes	n.s.	n.s.	Duplicate	5.0 pM	n.s.	1.0 µM	n.s.	Yes	Against in-house prepared PNP in each run
Efthymiou	2022	PPP	Citrated plasma, double centrifugation: 15 min., 2000 *g* at RT	−80°C	10 min. 37 °C WWB	Yes 1 : 1 (sample: PNP) if VKA therapy	1. Yes 2. No	1. n.s. 2. ST Genesia (Stago, France)	1. n.s. 2. ST Genesia	1. n.s. 2. Duplicate	1. 5.0 pM 2. n.s.	1. Synthetic 2. Recombinant human	1. 4.0 µM 2. n.s.	1. Synthetic 2. n.s.	1. n.s. 2. No	1. n.s. 2. Not applicable
Foret	2021	PRP	Citrated plasma, PRP: 10 min., 190 *g* at 20°C + addition of PPP (centrifugation: 10 min., 2000 *g* at 20°C) to obtain 150 G/L platelets	No storage (fresh PRP)	No storage	No	Yes	Fluoroskan Ascent (Thermo Fisher Scientific, Finland)	Thrombinoscope (Netherlands), version n.s.	Triplicate	n.s.	Recom-binant, human (Dade Behring, Germany)	n.s.	PRP.	No	Not applicable
Green	2012	PPP	Citrated plasma, double centrifugation: 15 min., 2000*g* at RT	−80°C	37°C, n.s.	No	No	ACL TOP500 (Werfen, Spain)	n.s.	Duplicate	1.5 pM	Recom-binant human (Recombiplastin 2 G) (Werfen, Spain)	10 µg/ mL	n.s.	Yes	Against commercial PNP (Techno-clone, Austria) in each run

(*continued*)

TABLE 2 Continued

First author	Year	Preanalytical					Thrombin generation methodology								
		PPP/ PRP	Sample preparation	Storage	Thawing	Mixing with PNP	CAT	Instrument	Software	Replicate amount	TF f.c.	TF origin	PL f.c.	PL origin	Nor-malised	Normalisation method
Hanly	2000	PPP	Citrated plasma, single centrifugation: 30 min., 3000*g*	−70°C	n.s.	Yes 1:2 (sample: PNP*)	No	In house chromogenic thrombin generation assay	n.s.	Triplicate	n.s.	Thrombo-plastin (Sigma, US)	n.s.	Thrombo-plastin (Sigma, US)	No	Not applicable
Kremers	2018	PPP	Citrated plasma, double centrifugation: 10 min., 2821 *g*	−80°C	n.s.	No	Yes	n.s.	Throm-binoscope (Nether-lands), version n.s.	n.s.	1.0 pM 5.0 pM	Recom-binant n.s. (Stago, France)	4.0 μM	Synthetic: 60 mol% DOPC, 20 mol % DOPS, and 20 mol% DOPE (n.s.)	No	Not applicable
Liestøl	2007	PPP	Citrated plasma: centrifugation 15 min., 2000g at 20 °C + filtration [Millex-GV 0,22 μm Filter Unite, Millipore, France]	−70°C; part of samples were transported at RT for 1-2 days between centrifu-gation and filtration	n.s.	Yes 1:1 (sample: PNP)	Yes	Fluoroskan Ascent (Thermo Fisher Scientific, Finland)	Throm-binoscope (Nether-lands), version n.s.	n.s.	5.0 pM	Recom-binant, human (Dade Behring, Germany)	4.0 μM	Synthetic: 60 mol% PC, 20 mol% PS, and 20 mol% PE (Avanti Polar Lipids, US)	Yes	Against in-house prepared PNP in each run
Matsumoto	2017	PPP	Citrated plasma, single centrifugation: 15 min., 1500 *g*	-80°C	37°C, n.s.	No	Yes	Fluoroskan Ascent (Thermo Fisher Scientific, Finland)	Throm-binoscope (Nether-lands), version n.s.	n.s.	0.5 pM 1.0 pM	Recom-binant, human (Dade Behring, Germany)	4.0 μM	n.s.	Yes	Against in-house prepared PNP in each run
Ramirez	2021	PPP	Citrated plasma, double centrifugation: 15 min., 2000*g* at RT	-80°C	10 min. 37 °C WWB	Yes 1:1 (sample: PNP) if VKA therapy	Yes	n.s.	Throm-binoscope (Nether-lands), version n.s.	n.s.	5.0 pM	Synthetic	4.0 pM	Synthetic	No	Not applicable
Zuily	2012	PRP	Citrated plasma, PRP: 10 min., 190 *g* at 20°C + addition of PPP (centrifugation: 10 min., 1750*g*) to obtain 150 × 10^9 platelets/L if platelet count in PRP >150 × 10^9/L	No storage (fresh PRP)	No storage	No	Yes	Fluoroskan Ascent (Thermo Fisher Scientific, Finland)	Throm-binoscope (Nether-lands), version n.s.	n.s.	0.5 pM	Recom-binant, human (Dade Behring, Germany)	n.s.	PRP	No	Not applicable

*PNP, but not platelet poor Abbreviations: CAT, calibrated automated thrombogram; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DOPS, dioleoyl phosphatidylserine; f.c., final concentration; n.s., not specified; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PNP, pooled normal plasma; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PS, phosphatidylserine; RT, room temperature; TF, tissue factor; US, United States; VKA, vitamin K antagonist; WWB, warm water bath.

10.3389/fcvm.2023.1075121

anticoagulant use in the APS patient population alone were not provided (42). Two studies only included APS patients and thrombotic controls on VKA treatment (35, 36). Both studies corrected for possible effects of anticoagulant therapy by mixing 1:1 with PNP. Bloemen et al. included three VKA-treated patients and two patients without anticoagulants to determine the effect of anticoagulant on TG (39). Three studies only included patients without anticoagulants or patients that had stopped treatment (38, 43, 48). In addition, two studies did not provide details regarding anticoagulant use in APS patients (44, 47).

Outcomes

Multiple parameters can be investigated when using a TG assay. Resistance to aPC is considered when there is less inhibition of TG than expected when activating the protein C pathway. Eight studies reported continuous data of results on which aPC-r assessment was based. They reported the average or median values with standard deviation (SD), 95% confidence interval (95% CI) or interquartile range (IQR) (**Table 3**). Three studies reported and compared the positivity rate or prevalence of aPC-r in APS and control subjects (**Table 4**) (36, 37, 43). ORs were calculated based on the available data for two studies (36, 43). aPC-r was assessed based on normalised inhibition of endogenous thrombin potential (ETP) (35, 46) or peak height (PH) (47), non-normalised inhibition of ETP (36, 37) or PH (45), ETP ratio (38, 42), normalised area under the TG curve (AUC) ratio (43), and aPC concentration needed to obtain 50% ETP inhibition (IC50-aPC) (48). Foret et al. found a significantly higher aPC-r in patients with APS compared to patients with the presence of aPL without clinical APS manifestations ($p = 0.04$), but not compared to a group of isolated AID patients without the presence of aPL (42). Arachchillage et al. observed a higher aPC-r, expressed as a lower normalised ETP inhibition with aPC and Protac®, in VKA-treated thrombotic APS patients compared to a VKA-treated control group and a healthy control group (35). Interestingly, they also observed a significantly different aPC-r between both control groups when using Protac®, but not if aPC was used (35). Efthymiou et al. demonstrated a significant association between aPC-r and primary APS patients compared to thrombotic controls when using TM, aPC, or Protac® with OR ranging from 6.8 to 12.8 (36). In contrast, no significant difference was observed in APS patients compared to SLE patients without thrombotic history ($p > 0.05$) (36). Similarly, Ramirez et al. could not demonstrate a significant difference in the prevalence of aPC-r between a group of secondary APS patients and SLE patients without a thrombotic history including both aPC and Protac® methods for aPC-r determination (37). Efthymiou et al. also compared the use of TM, aPC, and Protac® for determining aPC-r which showed poor agreement, but a comparable positivity rate based on in-house determined cut-off values (36). Kremers et al. showed that APS patients had significantly higher aPC-r compared to VKA treatment-matched controls, demonstrated by a lower ETP inhibition after adding TM (45). Liestøl et al. also demonstrated a higher level of aPC-r in VKA-treated and untreated APS patients compared to VKA-treated and untreated controls, respectively (46). Considering the overall APS group ($n = 52$), the median normalised ETP inhibition was significantly lower compared to a group of LA-positive patients without APS (44%, 95% CI: 30.1–55.7 vs. 78.8%, 95% CI: 73.9–95.8), corresponding to a higher aPC-r in APS patients (46). Similarly, Matsumoto et al. demonstrated a higher aPC-r in the LA-positive APS group compared to LA-positive controls, but with high variation within the groups (32). Zuily et al. showed that the IC50-aPC was higher in primary and secondary APS patients compared to presumably healthy controls (48). Although, non-APS SLE patients also showed a higher IC50-aPC (48). Billoir et al. observed that both thrombotic and obstetric APS patients had significantly higher aPC-r compared to healthy controls and aPL-carriers ($p = <0.001$) (38). This was also confirmed by Green et al. who demonstrated a higher rate of aPC-r in thrombotic APS patients compared to healthy controls (OR 5.9, $p = 0.005$), using a chromogenic substrate-based TG method (43).

As a second outcome, other TG parameters were defined such as PH or thrombin peak, ETP or AUC, lag time (LT), and time to peak (ttPeak) which can be derived from the thrombogram (12). These parameters can be reported as absolute results, but can also be normalised by dividing the patient result by a PNP result of the same run, reducing inter-run variability (15). Seven of the 14 retrieved articles described at least one of the parameters LT, ETP, ttPeak, or PH (**Table 5**) (38–41, 45–47). Only two studies normalised their data against PNP (41, 46). None of the seven studies reported reference values for the separate parameters. Therefore, only continuous data between patient and control groups could be compared. Kremers et al. could not demonstrate a significant difference in LT, ETP, PH, or ttPeak between APS patients and control groups using CAT with 5.0 pM TF as a trigger (45). When using 1.0 pM TF, no significant differences were observed comparing APS patients treated with VKA compared to a VKA-treated control group, although LT and PH were significantly higher in APS patients without VKA treatment compared to healthy controls (45). On the other hand, the same group found conflicting results in a more recent study (40). They used a developmental cohort consisting of 31 APS patients and 66 healthy controls to set up a neural network including TG and thrombin dynamics parameters. In this cohort, it was observed that APS patients had a shorter LT and ttPeak, decreased ETP and increased PH. In a second cohort, they also found a decreased ETP, and PH, but an increased LT, and ttPeak in APS patients compared to controls. In the second cohort, APS patients on VKA treatment were included, while they were excluded in the developmental cohort. Despite these discrepancies between both cohorts, the neural network was able to differentiate between APS patients and non-APS patients with an accuracy ranging from 73% to 93% depending on the control population considered (40). Liestøl et al. demonstrated a lower normalised ETP in VKA-treated LA-positive APS patients compared to VKA-treated controls (46). Interestingly, LA-positive controls without clinical APS criteria also showed a reduced normalised ETP compared to healthy controls ($p <$

Table 3 Summary of continuous outcomes of thrombin generation based resistance to activated protein C.

First author (year)	aPC-r method	Patient group (*n*)	aPC-r results patients	Control group (*n*)	aPC-r results controls	p-value	Remarks
A. Cross-sectional studies							
Foret (2021)	Ratio of ETP (CAT) with aPC and without aPC using PRP**	Primary and secondary APS (82)	0.583 +/- 0.278	1. AID controls (15)	1: not specified	>0.05	None
				2. aPL-carriers (20)	2: 0.423 +/- 0.239	0.04	
Arachchillage (2014)	Normalised inhibition (%) of ETP (CAT) with Protac and rhAPC using PPP 1:1 mixed with PNP*	VKA-treated TAPS (51)	Protac: 66.0% (59.5-72.6) rhAPC: 81.3% (75.2-88.3)	1. VKA-treated thrombotic controls (51)	1. Protac: 80.7% (74.2-87.2)	0.007	Comparing the two control groups: aPC-r with Protac significantly different between both control groups.
					1. rhAPC: 97.7% (93.6-102)	0.002	
				2. Healthy controls (51)	2. Protac: 102% (96.2-108)	<0.0001	
					2. rhAPC: 98.3% (92.2-104)	0.01	
B. Case-control studies							
Kremers (2018)	Median inhibition (%) of PH (CAT) with TM using PPP	A. VKA-treated APS (50)	A: 15%	1. VKA-treated controls (31)	1: 35%	A-1: <0.001	None
		B. APS without VKA (30)	B: 10%	2. Healthy controls (45)	2: 50%	B-2: <0.001	
Liestøl (2007)	Normalised inhibition (%) of ETP (CAT) with aPC using PPP 1:1 mixed with PNP*	A. VKA-treated APS (34)	A: 33.8% (28.8-55.7)	1. VKA-treated controls (38)	1: 115% (111-122)	A-1: <0.001	Overall patient group had median ETP inhibition of 44.6% (95%CI: 30.1-55.7) and was significantly different from control group 2 with LA presence (p=0.003).
				2. LA-positive controls (29)	2: 78.8% (73.9-95.8)	Not available	
		B. APS without VKA (18)	B: 52.0% (41.0-81.2)	3. Healthy controls (53)	3: 107% (106-108)	B-3: <0.001	
Zuily (2012)	APC concentration (nM) necessary for 50% ETP inhibition (CAT) after addition of aPC in PRP (=IC50-aPC) ***	Not applicable	Not applicable	1. SLE controls without aPL (13)	1: 27.3 nM (23.5-43.5)	Not available	IC50-aPC of SLE controls without aPL presence was also significantly increased compared to controls.
		A. Primary APS (38)	A: 15.3 nM (9.7-34.0)	2. Controls, not specified (39)	2: 10.4 nM (8.5-15.8)	A-2: <0.05	
		B. Secondary APS (10)	B: 64.1 nM (25.9-65.0)			B-2: <0.05	
Billoir (2021)	Ratio (%) of ETP (CAT) with aPC and without aPC using PPP**	A. TAPS (19)	A: 52% +/- 16.4	1. aPL-carriers (11)	1: not specified	A-1: <0.001	Significant difference between patient groups and aPL-carriers, but values not mentioned.
						B-1: not specified	
		B. OAPS (11)	B: 64.1% +/- 14.6	2. Healthy controls (25)	2: 27% +/- 13.8	A-2: <0.001	
						B-2: <0.001	
Green (2012)	Normalised ratio of AUC from TG assay with and without Protac in PPP***	TAPS without anticoagulation (17)	1.1 (0.8-1.4)	Healthy controls (35)	2.8 (2.4-4.7)	<0.001	Other thrombotic patients with protein C/S deficiency or FV Leiden had also significantly different normalised AUC ratio compared to healthy controls.
Matsumoto (2016)	Normalised inhibition (%) of PH (CAT) with aPC using PPP**	APS, LA-positive (10)	5% +/- 7	1. LA-positive controls (10)	1. 42% +/- 39	1. <0.01	The normalised inhibition of PH by aPC was also significantly lower in LA-positive controls compared to healthy controls.
				2. Healthy controls (not specified)	2. Not specified	2. <0.01	

*Median (95% CI);
**Mean +/- SD;
***Median (IQR)

Abbreviations: AID, autoimmune disease; aPC, activated protein C; aPC-r, activated protein C resistance; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; AUC, area under the thrombin generation curve; CAT, calibrated automated thrombography; ETP, endogenous thrombin potential; FV, Factor V; LA, lupus anticoagulant; OAPS, obstetric APS; PH, peak height; PNP, pooled normal plasma; PPP, platelet-poor plasma; PRP, platelet-rich plasma; rhAPC, recombinant human activated protein C; SLE, systemic lupus erythematosus; TAPS, thrombotic APS; TG, thrombin generation; TM, thrombomodulin; VKA, vitamin K antagonist; VTE, venous thromboembolism.

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Table 4 Prevalence and positivity rate of thrombin generation-based resistance to activated protein C (dichotomous outcome).

First author (year)	aPC-r method	Cut-off value	Patient group (*n*)	Patients with aPC-r	Control group (*n*)	Controls with aPC-r	Odds ratio (95% CI)	p-value
A. Cross-sectional studies								
Efthymiou (2022)	Inhibition (%) of ETP on PPP with: A. Protac (CAT); B. rhAPC (CAT); C. TM (ST Genesia). Mixed 1:1 with PNP if VKA treatment	99th percentile: A. <63% B. <56% C. <49%	Primary APS (106): 83 TAPS and 23 OAPS	A: 67/106 B: 61/106 C: 57/106	Thrombotic control, no APS or inherited thrombophilia (36)	A: 5/36 B: 6/36 C: 3/36	A: 10.7 (3.83-29.7) B: 6.78 (2.60-17.7) C: 12.8 (3.70-44.3)	A: <0.0001 B: 0.0001 C: 0.0001
					SLE patients without thrombotic history (37)	A: 17/37 B: 19/37 C: 17/37	A: 2.02 (0.95-4.30) B: 1.28 (0.61-2.72) C: 1.37 (0.65-2.90)	A: 0.07 B: 0.51 C: 0.42
Ramirez (2021)	Inhibition (%) of ETP (CAT) with Protac and rhAPC using PPP, mixed 1:1 with PNP if VKA treatment	99th percentile of 100 healthy subjects: rhAPC <56%; Protac <63%	Secondary APS (24)	Not specified	SLE with aPL without thrombotic history (26)	Not specified	Not available	Not significant
					SLE without aPL (n = 14)			
					Isolated SLE (n=37)			
B. Case-control studies								
Green (2012)	Normalised ratio of AUC from TG assay with and without Protac in PPP	5th percentile of 35 healthy subjects	TAPS without anticoagulation (17)	11/17	Patients with history of thrombosis and FV Leiden (19)	19/19	0.045 (0.002-0.88)	0.04
					Patients with history of thrombosis and with Protein C or S deficiency (9)	8/9	0.23 (0.02-2.3)	0.21
					Thrombotic control, no APS or inherited thrombophilia (42)	10/42	5.87 (1.73-19.9)	0.005

Abbreviations: aPC-r, activated protein C resistance; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; AUC, area under the thrombin generation curve; CAT, calibrated automated thrombography; ETP, endogenous thrombin potential; FV, Factor V; OAPS, obstetric APS; PNP, pooled normal plasma; PPP, platelet poor plasma; rhAPC, recombinant human activated protein C; SLE, systemic lupus erythematosus; TAPS, thrombotic APS; TM, thrombomodulin; TG, thrombin generation; VKA, vitamin K antagonist.

0.001) (46). In contrast, Billoir et al. demonstrated a higher ETP for both thrombotic and obstetric APS patients compared to healthy controls using the CAT method and 1.0 pM TF as an activator (38). When compared to aPL-positive controls (without clinical APS criteria), only obstetric APS patients had significantly higher ETP values, whereas thrombotic APS patients did not. They also showed that thrombotic and obstetric APS patients had a higher PH compared to both healthy controls and aPL-carriers (38). Devreese et al. previously demonstrated that a ratio of PH/LT instead of assessing the parameters separately might be more useful (25). In the study included here, they reported a pilot study that demonstrated that LA-positive thrombotic APS patients displayed a lower PH/LT ratio compared to LA-positive controls, thrombotic controls, and healthy controls (41). Furthermore, Matsumoto et al. described a longer LT in ten LA-positive APS patients compared to a LA-positive control group, but with similar PH levels (47). Bloemen et al. also described a longer LT in APS patients compared to healthy controls, although the patient and control group only consisted of five subjects each (39). Results were summarised in an effect direction plot in **Figure 2**.

The outcomes of one study could not be included in one of the proposed groups (44). They used a chromogenic substrate to determine the inhibition of TG by comparing the thrombin generated in patients to healthy controls and expressing results as standard error of mean or z-score. Inhibition of the *in vitro* TG was more significant in APS patients ($n = 29$) compared to control patients without thrombotic or obstetric complications of APS ($n = 30$). When applying a cut-off of $|z| = 2$, the OR for APS diagnosis was 5.43 (95% CI 1.76–16.8) (44).

Discussion

The diagnosis of APS predominantly relies on a combination of laboratory assays to measure the presence of aPL. However, these laboratory tests still show methodological and diagnostic shortcomings and a lack of standardisation (50). The laboratory tests for the detection of aPL comprise clotting-based assays for the detection of LA and solid-phase immunoassays for the detection of aCL and aβ2GPI IgG/IgM antibodies (3). TG assays measure the dynamic process of *in vitro* thrombin generated over time and offer a more global assessment of the coagulation compared to traditional coagulation assays (9). In addition, several studies have investigated the role of TG as a new tool to assess coagulation in patients with a wide variety of coagulation disorders (11, 14). This review was performed to assess if TG assays could be used as a diagnostic tool for, on one hand, the laboratory diagnosis of APS patients and, on the other hand, for identifying APS patients at high risk for recurrent clinical manifestations.

Table 5 An overview of thrombin generation parameters.

First author (year)	Parameter (unit)	Patient group (*n*)	Patient results	Control group (*n*)	Control results	P-value	
Kremers (2018)	LT (min) ETP (nM●min) PH (nM) ttPeak (min)	A: VKA-treated APS (50)	Graphical representation only	1. VKA-treated controls (31)	Graphical representation only	A-1: n.s.	
		B: APS without VKA (30)		2. Healthy controls (45)		B-2: LT longer ($p<0.001$) and PH higher ($p<0.01$) in APS when using 1 pM TF, not when using 5 pM TF. ETP and ttPeak n.s.	
De Laat – Kremers (2021)	LT (min) ETP (nM●min) PH (nM) ttPeak (min)	A: Development cohort: APS (31)	Graphical representation only	1. Development cohort: Healthy controls (66)	Graphical representation only	A-1: APS: shorter LT ($p<0.01$) and ttPeak, decreased ETP and increased PH compared to controls ($p<0.05$)	
		B: Validation cohort: APS (42)	Not available	2. Validation cohort: Healthy controls (38)	Not available	B-2: APS: increased LT, decreased ETP, longer ttPeak, and decreased PH in APS patients compared to controls ($p<0.05$)	
Liestøl (2007)	ETP, w/o aPC (normalised) ***	A: VKA-treated APS (34)	0.63 (0.59-0.66)	1. VKA-treated controls (38)	0.73 (0.70-0.77)	A-1: <0.001	
	ETP, with aPC (normalised)***		3.69 (2.38-4.50)		0.17 (0.13-0.28)	A-1: Not reported	
	ETP, w/o aPC (normalised) ***	B: APS without VKA (18)	0.94 (0.83-1.00)	2. Healthy controls (53)	1.01 (0.96-1.05)	B-2: <0.001	
	ETP, with aPC (normalised)***		2.61 (2.02-5.04)		0.58 (0.54-0.72)	B-2: Not reported	
	ETP, w/o aPC (normalised) ***	Not applicable	Not applicable	3. LA-positive (29)	0.85 (0.81-0.93)	Not reported	
	ETP, with aPC (normalised)***				1.92 (1.22-2.81)		
Devreese (2010)	PH/LT ratio (nM/min)*	A: TAPS (8)	29.8 +/- 33.6	1. LA-positive (8)	90.7 +/- 29.7	A-1: 0.003	
				2. Thrombotic controls (21)	128 +/- 48.5	A-2: 0.0002	
				3. Healthy controls (25)	170 +/- 46.4	A-3: <0.0001	
Billoir (2021)	LT (min)*	A: TAPS (19)	Not reported	1. aPL-carriers (11)	13.6 +/- 3.9	A/B-1: Not reported	
	ETP (nM.min)**		1265 (956-1741)		Not reported	A-1: 0.08	B-1: 0.002
	PH (nM)**		153 (109-215)		Not reported	A-1: 0.048	B-1: 0.001
	LT (min)*	B: OAPS (11)	Not reported	2. Healthy controls (25)	4.89 +/- 1.65	A/B-2: Not reported	
	ETP (nM.min)**		1863 (1434-2080)		808 (756-853)	A-2: <0.001	B-2: <0.001
	PH (nM)**		254 (232-289)		78 (74-86)	A-2: <0.001	B-2: <0.001

(*continued*)

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Table 5 Continued

First author (year)	Parameter (unit)	Patient group (*n*)	Patient results	Control group (*n*)	Control results	P-value
Matsumoto (2017)	LT (min)*	A: LA-positive APS (10)	28.8 +/- 11.8	1. LA-positive (10)	12.5 +/- 7.7	A-1: <0.01
	PH (nM)*		158 +/- 99		158 +/- 75	A-1: Not reported
	LT (min)*	Not applicable	Not applicable	2. Healthy controls (25)	4.5 +/- 0.3	A-2: <0.01
	PH (nM)*				362 +/- 23	A-2: Not reported
Bloemen (2016)	LT (s)**	APS (5)	6.0 (5.15-7.85)	Healthy controls (5)	2.0 (1.75-2.25)	0.008

*Mean +/- standard deviation,
**Median (IQR),
***Median (95% CI);
Abbreviations: aPC, activated protein C; aPL, antiphospholipid antibodies APS, antiphospholipid syndrome; CI, confidence interval; ETP, endogenous thrombin potential; IQR, interquartile range; LA, lupus anticoagulant; LT, lag time; n.s., not significant; OAPS, obstetric APS; PH, peak height; TAPS, thrombotic APS; TF, tissue factor; ttPeak, time to Peak; VKA, vitamin K antagonist; w/o, without

Our review identified 1,160 records when searching the key concepts "thrombin generation" and "antiphospholipid syndrome". Fourteen articles were included that demonstrated TG data in APS patients. All included publications described retrospective case-control or cross-sectional studies. Interestingly, most of the studies (10/14) measured aPC-r in APS patients using the TG method. Resistance to aPC occurs due to a decreased inhibition of activated coagulation factor V (FVa) by aPC and is an important risk factor for venous thrombosis. aPC-r may occur due to various causes, either inherited (e.g., FV Leiden) or acquired (e.g., use of oral contraceptives) (51). Traditionally, laboratory tests to screen for the aPC pathway are based on the activated partial thromboplastin time (aPTT). In this test, aPC-r is considered when the prolongation of the aPTT by adding aPC is less than expected (52). However, interpretation of aPTT-based aPC-r can be complicated by a prolonged aPTT at baseline, often observed in aPL-positive patients (53). Alternatively, TG-based aPC-r testing could be performed in these patients to overcome the issues associated with aPTT-based aPC-r tests. Furthermore, TG assays might identify patients with resistance to aPC-r that cannot be detected with the traditional assays (54). Nevertheless, there is a need for standardisation of TG-based aPC-r assays, since methodological variation between studies is large. Variation in TG-based aPC-r assays occurs mostly on two levels. First, an exogenous substance needs to be added to the TG reaction to evaluate the aPC pathway as the amount of endogenous aPC formed is too low in a standard TG assay (14). In this review, three exogenous substances were described, namely aPC, Protac®, and TM. Both Protac® (enzymatic) and TM (thrombin cofactor) lead to endogenous protein C activation meaning that the function of endogenous protein C is examined in contrast to when exogenous aPC is added. Secondly, different strategies can be used to compare TG before and after adding the substance for aPC pathway evaluation. Generally, a ratio of the PH or ETP, either normalised with PNP or not, before and after adding the aPC substance is assessed.

The diversity in TG-based aPC-r methods and reporting of the results complicated the general interpretation of the studies in this review, although some conclusions may be drawn. It appears that aPC-r is higher in patients with thrombotic APS compared to healthy controls, but also compared to thrombotic controls without inherited thrombophilia. This also seems to be true for obstetric APS patients, although only two studies analysed obstetric APS patients as a separate group and the total number of patients was relatively small (36, 38). Treatment with VKA did not influence the interpretation as also VKA-treated APS patients showed higher aPC-r, both compared to VKA-treated control groups and healthy controls. Most studies did however attempt to correct VKA-related coagulation factor deficiencies by 1 : 1 mixing of patient plasma with PNP. Interestingly, in one study a VKA-treated control group showed significantly higher aPC-r compared to healthy controls when using Protac®, but not when using aPC even after 1:1 mixing of the samples with PNP (35). The same trend was observed in two other studies, where aPC-r was significantly higher in VKA-treated controls compared to

		Control group	aPC-r				LT				ETP				PH				ttPeak			
			A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
A. Cross-sectional studies																						
First author (year)	**NOS score**	**Patient group**																				
Efthymiou (2022)	4	APS		▲	◄►																	
		TAPS																				
		OAPS																				
Foret (2021)	4	APS	▲		◄►																	
		TAPS																				
		OAPS																				
Arachchillage (2014)	4	APS																				
		TAPS		▲		▲																
		OAPS																				
Ramirez (2021)	3	APS																				
		TAPS			◄►																	
		OAPS																				
B. Case-control studies																						
First author (year)	**NOS score**	**Patient group**																				
Kremers (2018)	7	APS		▲		▲		◄►		▲*|◄►**		◄►		◄►		◄►		▲*|◄►**		◄►		◄►
		TAPS																				
		OAPS																				
De Laat-Kremers (2021)	6	APS (1)|(2)								▼|▲				▼|▼				▲|▼				▼|▲
		TAPS																				
		OAPS																				
Liestøl (2007)	6	APS	▲	▲		▲						▼		▼								
		TAPS																				
		OAPS																				
Devreese (2010)	6	APS																				
		TAPS																				
		OAPS																				
Zuily (2012)	5	APS				▲																
		TAPS																				
		OAPS																				
Billoir (2021)	5	APS																				
		TAPS				▲					◄►			▲	▲			▲				
		OAPS				▲					▲			▲	▲			▲				
Hanly (2000)	5	APS																				
		TAPS																				
		OAPS																				
Green (2012)	5	APS																				
		TAPS				▲																
		OAPS																				
Matsumoto (2017)	5	APS	▲			▲	▲			▲					◄►			▼				
		TAPS																				
		OAPS																				
Bloemen (2016)	3	APS								▲												
		TAPS																				
		OAPS																				

FIGURE 2
Effect direction plot comparing different outcomes between patient groups and control groups. Explanation: ▲: increased in patient population compared to control population; ▼: decreased in patient population compared to control population; ◄►: no significant difference between patient and control population. Control populations: (A): antiphospholipid antibody carriers; (B): thrombotic controls and control patients on vitamin K antagonist therapy; (C): autoimmune disease control group; (D): presumably healthy controls. *Thrombin generation with 1 pM TF, **5 pM TF; (1) developmental cohort, (2) validation cohort. Abbreviations: aPC-r, activated protein C resistance; APS, antiphospholipid syndrome; ETP, endogenous thrombin potential; LT, lag time; NOS, Newcastle-Ottawa Score; OAPS, obstetric APS; PH, peak height; TAPS, thrombotic APS; ttPeak, time to peak.

healthy controls when using TM (45) but not with aPC (46). This may be explained by the impaired production of endogenous protein C due to VKA, suggesting that it might be useful to assess underlying aPC-r using aPC instead of TM or Protac® in VKA-treated individuals. Tightly matching patients and controls for anticoagulant treatment should be imperative in investigating the value of TG in APS patients. Unfortunately, only limited information on anticoagulant treatment was provided in the included publications which is a limitation in this review. Three studies included anticoagulant-matched controls. Those studies were matched based on anticoagulant type, but not on intensity of treatment (35, 45, 46).

The prevalence and degree of aPC-r does not seem to differ between patients with primary or secondary APS and patients with SLE even without circulating aPL or a history of clinical manifestations of APS (36, 37, 42, 48). SLE patients with aPC-r might be at higher risk for thrombosis compared to those without aPC-r. This was outside the scope of the review and should be addressed by prospective studies. It is known that the prevalence of circulating non-criteria aPL such as antiphosphatidylserine/prothrombin antibodies (aPS/PT) is higher in patients with AID compared to the general population (55). Presence of these aPL might partially explain comparable aPC-r between APS and SLE (or other AID) patients (56). This raises the question whether the observed aPC-r is an *in vitro* finding due to the presence of aPL or really associated with an increased risk of thrombo-embolic complications. Of note, other mechanisms than presence of aPL leading to impaired protein C activation might be present in AID patients (57).

Information on ethnicity of the included cases was not available in all but one publication. Nevertheless, information regarding ethnicity might be important, since various studies have demonstrated ethnic variation in coagulation parameters and risk for venous and/or arterial thrombosis (58, 59).

In only a few studies included in this review, thrombogram-derived parameters were reported, and mostly in a selective manner introducing considerable selection bias. In most studies no normalisation procedure of data was performed which might lead to a decreased standardisation and comparability between studies (15). Compared to healthy controls, only two studies

reported a prolonged LT in APS patients (39, 47) and two studies were conflicting as they each included two patient populations with different LT results (40, 45). In addition, PH and ETP values were highly variable and inconsistent results were reported between studies. This discrepancy may partially be explained by the hypothesis that distinct aPL profiles may be associated with different TG profiles (60). Furthermore, to fairly evaluate the predictive ability of TG in relation to the risk of thrombotic or obstetric manifestations, it is necessary to calculate an OR, or to investigate the sensitivity, specificity, and positive and negative predictive values of the assay. Unfortunately, this was impossible because none of the studies reported a predefined cut-off value for any of the TG-derived parameters. Moreover, it might be more interesting to combine different TG parameters for the diagnostic evaluation of APS patients. Devreese et al. showed that the ratio of PH/LT could partially discriminate LA-positive APS patients from LA-positive patients without thrombosis although this has not been verified in an independent study (41). The study of de Laat-Kremers et al. showed that combining TG parameters and thrombin dynamics might accurately identify APS patients using an artificial intelligence approach (40), but this has to be verified in independent larger cohorts. Furthermore, thrombin dynamics is a technique that is not routinely available (40). It cannot be excluded that TG might be useful as a diagnostic marker as aPL showed direct influence on the TG profile based on spiking experiments (25, 56). This review has demonstrated that there is a lack of evidence on how deviant TG results correlate with a higher thrombotic or obstetric risk in APS patients as no prospective studies were available.

The total amount of thrombin activity over time can be measured using multiple methods, including fluorogenic and chromogenic substrate-based TG methods. Although both methods measure TG, there are significant methodological differences between the two assays. These differences originate from the substrates themselves and the sample preparation needed for each substrate. Fluorogenic substrate-based assays utilise the 7-Amino-4-methylcoumarin (AMC) fluorophore, whereas the chromogenic substrate-based assays use the para-nitroaniline (pNA) chromophore (61, 62). Chromogenic substrates require defibrinated plasma since fibrin can cause turbidity which is known to interfere with the absorption (61). However, the removal of fibrinogen has a profound effect on the TG curves by reducing the PH and increasing the formation of thrombin-α_2M complexes (62). Finally, the onset of TG is significantly faster in TG assays that utilise a chromogenic substrate compared to assays using a fluorogenic substrate (61). These differences make the TG data from chromogenic and fluorogenic substrate-based techniques hard to compare. Even within the fluorogenic substrate-based methods, ST Genesia® and CAT methodologies showed poor agreement for detecting aPC-r, although a different methodology was used for protein C activation (TM and Protac® or recombinant aPC, respectively) (36). Another study investigating patients with liver disease also demonstrated significant differences between ST Genesia® and CAT analysis (63). Further studies are needed to investigate whether automated TG analysers such as ST Genesia® are adequate for replacing the traditional CAT methodology.

Other differences in the TG protocol also influenced the comparability between studies. Although most included studies in this review used largely identical CAT-TG protocols, four studies used lower TF concentrations. At low TF concentrations, coagulation factors from both the intrinsic and extrinsic pathways influence the TG assay (64), whereas at high TF concentrations, the TG assays are only influenced by the factors of the extrinsic pathway (64). Furthermore, the addition of synthetic phospholipids has been shown to strongly influence all TG parameters (65). Therefore, differences in phospholipid concentration could severely impact the comparability of studies. Overall, following a standardised protocol for conducting and reporting TG research is very important to fairly compare outcomes between different studies. The International Society on Thrombosis and Haemostasis – Scientific and Standardization Committee on Lupus Anticoagulant/Antiphospholipid Antibodies recently published recommendations on the measurement of TG, aiming for an increased standardisation (15), in addition to previous guidelines on platelet-dependent TG (66). No formal heterogeneity assessment was performed, but we can informally conclude that the studies included in this review are extremely heterogenous. While the development of automated TG systems should increase the inter-laboratory harmonisation and standardisation of the TG assay, efforts should be undertaken to follow the international recommendations (15, 66). Recommendations on TG-based aPC-r measurement could benefit harmonisation, as the currently investigated methods are very heterogeneous across studies.

In addition, this study only included patients with APS, diagnosed based on the presence of the aPL that are included in the Sydney classification criteria (LA, aCL, and aβ2GPI) (1). Information on the assays that were used for aPL detection were not extracted from the studies, although APS classification may depend on the type of assay used (67, 68). Whether including non-classification aPL such as aPS/PT may help to uniformly characterise and classify APS patients or might help to decrease the heterogeneous outcomes observed in this systematic review, is questionable. The more that in the upcoming new classification criteria aPS/PT is not included (69). However, for diagnostic reasons, aPS/PT might help in subpopulations, such as those where LA measurement is hampered by interference of anticoagulation. We acknowledge that there is no gold standard for diagnosis of APS and we rely on classification criteria for conducting research in APS patients. Nevertheless, a prospective study not included in this review identified TG-based aPC-r as a higher risk factor for thrombosis in a population of patients with APS and/or SLE and aPL carriers, compared to the traditional Sydney criteria aPL (33). However, standard-of-care anticoagulant or antiplatelet therapy was initiated in patients based on the treating physicians' assessment introducing bias as patients with a higher risk profile, including the presence of (multiple) aPL, will most likely lead to a higher level of anticoagulation treatment in those patients and thus lower the incidence of thrombosis. This shows that there are both advantages and disadvantages to limiting systematic reviews to

studies with patients categorised according to the APS classification criteria as the population of interest.

The goal of this review was to investigate the value of TG assays in the diagnosis and risk stratification of APS. No prospective cohort studies were identified in this review and therefore no information could be synthesised on the obstetric or thrombotic risk associated with abnormal TG patterns in primary and secondary APS patients. However, higher aPC-r values and aPC-r prevalence are observed in APS patients compared to healthy and thrombotic controls, but the diagnostic and prognostic value is unclear compared to current diagnostic strategies. Results of other thrombogram-derived parameters such as LT and PH were conflicting across studies and more research is needed to identify their potential role in APS diagnosis. Publications on TG studies in APS were very heterogeneous in the applied TG methodology, preanalytical variables, and result description. Following the available guideline documents for reporting TG studies might improve harmonisation. Additional guidelines are needed for selection of the adequate TG methodology in APS studies.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

Author contributions

KD conceptualised the study; RG and AV designed the search strategy, independently screened all titles and abstracts, and full-text articles, and performed the study quality assessment of all included articles; KD acted as the third reviewer in case of disagreement; RG and AV performed the data extraction and synthesis; All steps were critically reviewed by BDL and KD; RG and AV wrote the initial version of the manuscript. BDL and KD provided critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

BDL and RG are employees of Synapse Research Institute, which is a part of the Stago group that markets the Calibrated Automated Thrombography and ST Genesia®.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1075121/full#supplementary-material.

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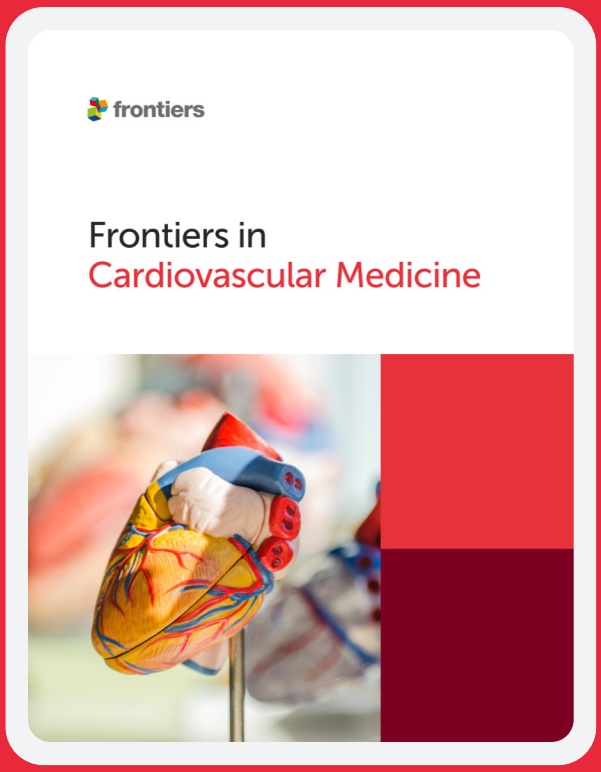

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