
Article:
Vries, MJA, Macrae, F orcid.org/0000-0002-7092-0422, Nelemans, PJ et al. (7 more authors) (2019) Assessment and determinants of whole blood and plasma fibrinolysis in patients with mild bleeding symptoms. Thrombosis Research, 174. pp. 88-94. ISSN 0049-3848

https://doi.org/10.1016/j.thromres.2018.12.004

Assessment and determinants of whole blood and plasma fibrinolysis in patients with mild bleeding symptoms

Running title

Assessment of fibrinolysis in mild bleeders

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Trial registry

Registry: Nederlands Trial Register (NTR)

Trial ID: (ntr)4070

Title: Preoperative evaluation of bleeding risk during surgery

Abstract

Enhanced clot lysis is associated with bleeding, but assessment of lysis capacity remains difficult. The plasma turbidity lysis and whole blood tissue Plasminogen Activator-Rotational Thromboelastometry (tPA-ROTEM) assays estimate fibrinolysis under more physiological conditions than clinically used assays. We hypothesized that these assays could find signs of *enhanced* lysis capacity in patients who report bleeding symptoms, but are not diagnosed with bleeding disorders. We also aimed to gain insight in determinants of the results of these lysis assays. Data from 240 patients with and 95 patients without self-reported bleeding symptoms were obtained, who were included in a study that primarily aimed to assess prevalence of haemostatic abnormalities in preoperative patients. ROTEM and turbidity assays were performed with rtPA. Blood counts, fibrinolysis and coagulation factor activities were determined. Data were analysed using multivariable linear regression models. Remarkably, patients reporting bleeding symptoms showed signs of significantly *impaired* lysis capacity in the tPA-ROTEM, but not in the turbidity lysis assay. In these patients, the tPA-ROTEM results depended on FII, FXII, plasminogen, α2-antiplasmin, PAI-1 and TAFI levels. The turbidity lysis results were significantly influenced by fibrinogen, α2-antiplasmin, PAI-1 and TAFI. In conclusion, the tPA-ROTEM and the turbidity lysis assay could not detect enhanced fibrinolytic capacity in patients with bleeding symptoms. This suggests that these symptoms are not caused by enhanced fibrinolytic activity. As both assays were sensitive to important determinants of fibrinolysis they may be able to detect a fibrinolytic imbalance, but this needs to be validated in patients with known hypo- or hyperfibrinolytic disorders.

Key words

Fibrinolysis, Bleeding, Diagnostic Techniques, Rotational Thromboelastometry, Turbidity

Introduction

The formation of a stable fibrin clot is essential for normal haemostasis. This requires mechanical stability and resistance to premature lysis.[1-3] Enhanced clot lysis has been associated with bleeding, demonstrated by the moderate to severe bleeding phenotype observed in patients with fibrinolysis inhibitor protein deficiencies such as plasminogen activator inhibitor 1 (PAI-1) and α2-antiplasmin (α2-AP).[4,5] Enhanced lysis has also been observed in Haemophilia patients, who form loosely-woven fibrin clots that are more prone to lysis.[3,6] Clot architecture has been shown to be a key determinant of clot lysis and is influenced primarily by local thrombin concentration.[1,3] Therefore, a functioning coagulation system leading to sufficient thrombin generation is essential for clot lysis resistance. A high thrombin concentration leads to the creation of dense clots with thin fibrin fibres, hindering plasminogen and its activators to penetrate, bind to fibrin fibres and lyse the clot.[2,3] Other factors that influence clot architecture include fibrinogen concentration and function, reactivity of platelets and the biochemical environment (e.g. calcium concentration and pH).[1,3] More specifically, platelets contain PAI-1 and α2-AP in their α-granules, as well as polyphosphate in the δ-granules, which modulate haemostasis and fibrinolysis.[7,8]

Since clot structure and lysis are influenced by several components, some of which are released locally by endothelial cells and platelets, investigating *in vivo* clot lysis potential is challenging. This is the main reason why reliable clot lysis assays are still lacking.[1,9] The fact that up to 70% of all patients with a mild clinical bleeding phenotype are never diagnosed[10,11], might be partly contributable to the shortcomings of currently used lysis assay. The classic Euglobulin Lysis Time (ELT) is still frequently used in the clinic, despite its major drawbacks. Essentially, it can investigate the function of plasminogen, plasminogen activators and fibrinogen, but not the influence of plasmin inhibitors or other haemostatic proteins on lysis.[12] Moreover, it does not include the formation of a fibrin clot formed under physiological conditions as substrate for the fibrinolytic enzymes. In the more recently developed lysis assays, the turbidity lysis assay and the tissue Plasminogen Activator-Rotational Thromboelastometry (tPA-ROTEM), all plasma proteins are present and fibrin is formed under more physiological conditions for the measurement of fibrinolysis. In the plasma based turbidity lysis assay, while all plasma components are included, platelets and other whole blood components are not and therefore cannot contribute to the lysis process. In comparison, the tPA-ROTEM is a whole blood assay and therefore coagulation factors, fibrinolysis proteins, platelets and other cells all may contribute to the assessment of clot lysis.[13,14]

We hypothesized that clot lysis capacity is enhanced in patients with mild bleeding symptoms. Therefore, this paper aimed to explore whether patients with bleeding symptoms showed signs of faster fibrinolysis than patients without bleeding symptoms. In addition, we studied the determinants of a plasma and whole

blood lysis assay in order to investigate their ability to detect any imbalances leading to hyperfibrinolysis.

Materials and methods

Study population and design

Data were obtained from a clinical observational study that primarily aimed to estimate and compare the prevalence and severity of haemostatic abnormalities in patients with and without reported bleeding symptoms on a guideline-based preoperative questionnaire.[15] The study population was recruited from consecutive patients who were scheduled for any kind of elective surgery in the Maastricht University Medical Centre in the period from September 2013 to January 2016 (Figure S1). Subjects eligible for inclusion were ≥18 years old, not diagnosed with bleeding disorders and did not use antithrombotic drugs or non-steroidal anti-inflammatory drugs. Patients with thrombocytopenia ($<100x10^9$/L), anaemia (haemoglobin level <13.2 g/dL for men, <11.8 g/dL for women) and pregnant women were excluded.

All patients completed a preoperative anaesthesiology bleeding questionnaire by themselves. Both patients reporting ≥1 bleeding symptoms on this questionnaire (bleeding after surgery, dental extraction or delivery, spontaneous haematomas, spontaneous nosebleeds, spontaneous gum bleeds, prolonged bleeding from minor wounds, menorrhagia or family members with clotting disorders), as well as a randomly selected sample of preoperative patients who did not report bleeding symptoms on this questionnaire were included. We primarily aimed to include all

patients who reported ≥1 bleeding symptoms on this questionnaire ("positive questionnaire"), and a sample of patients who did not report bleeding symptoms ("negative questionnaire"). Blood was drawn preoperatively, during the study visit. The following haemostatic assays were performed: fibrinogen levels, factor II, V, VII, VIII, IX, X, XI, XII, XIII activity levels, von Willebrand antigen and activity levels, light transmission aggregometry with 6 agonists and tPA, PAI, and antiplasmin activity levels. In 9% of patients reporting bleeding symptoms and 10% of patients not reporting bleeding symptoms a haemostatic abnormality was found (10 mildly reduced coagulation factor activities, 4 reduced von Willebrand factor activities, 16 platelet function defects and one increased tPA level; see Table S1). By far most abnormalities were mild, would not prompt clinical treatment and could not (completely) explain the bleeding symptoms.[15] Therefore, we decided to study clot lysis in all patients rather than excluding patients with these mild abnormalities.

This study complied with the Declaration of Helsinki and was approved by the local medical ethics committee, with all patients giving written informed consent.

Blood collection, preparation and storage

Between 9:00-14:30 hours venous blood was collected by venepuncture of the antecubital vein using 21-gauge needles and vacuum tubes, after at least 20 minutes of no physical activity. The tourniquet pressure was released during the filling of the first blood tube, which was not used for the haemostatic tests to avoid undesired platelet or coagulation activation.

Platelet free plasma (PFP) was obtained by centrifugation of citrated blood at 2,500 g for 5 min and then at 10,000 g for 10 min at 18℃. For all haemostatic tests

citrated blood was used (3.2% sodium citrate, Vacuette tubes, Greiner bio-one; except for ELT and tPA activity: Trinilyze Stabilyte Tubes). Complete blood count (EDTA 7.2 mg, BD Vacutainer, Plymouth), fibrinogen measurements and the ROTEM were performed within 2 hours of blood collection. All other tests were performed batch-wise in stored PFP (-80°C), which was frozen in aliquots within 2 hours of blood collection.

Laboratory measurements

Fibrinogen (Clauss method, Thrombin Reagent, Siemens) and FXIII activity (FXIIIact/subs, Siemens) measurements were performed on a Sysmex CS 2100i. FII, (Neoplastine R), FXI, FXII activity (CK Prest were performed by clotting assays using the specific factor-depleted plasma on Sta-R, (Stago, Paris). Plasminogen and α2-AP levels were measured by the use of chromogenic assays (Stachrom Plasminogen, Stachrom Antiplasmin, Stago, Paris). Bio-immunoassays were used to measure tissue plasminogen activator (tPA) activity, plasminogen activator inhibitor-1 (PAI-1) activity and thrombin activatable fibrinolysis inhibitor (TAFI) zymogen (Zymutest, Hyphen, Biomed).

The tPA-ROTEM was performed according to published methods[13], within 2 hours of blood withdrawal. Tissue Factor, $CaCl_2$ and rtPA were added to the whole blood (to obtain final concentrations of 35 pM TF, 10 mM $CaCl_2$ and 125 ng/mL rTPA). The device temperature was set to 37 ℃ and maximum runtime was 120 minutes. The following parameters were analysed: CT (clotting time), CFT (clot formation time), MCF (maximum clot firmness), LOT (lysis onset time; time taken for

amplitude to decrease by 15% of MCF), LT (lysis time; time taken for amplitude to drop to 10% of MCF), and delta (lysis speed, %/min between LOT and LT) (Figure 1A).

The turbidity and lysis assay was carried out as previously described by Scott et al. with a few minor changes.[16] In short, plasma samples were studied by turbidity on low binding microtiter well plates (Greiner Bio-One Ltd., Stonehouse, UK) using a Spectra Max plus 384 microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).[17] Plasma samples were diluted 1:6 in TBS (100mM NaCl and 50mM Tris, pH 7.4) and the reaction was initiated by addition of $CaCl_2$ (5 mM) and thrombin (Calbiochem; 0.5 U/mL, final concentrations). For fibrinolysis measurements, rtPA (85 ng/mL final concentration) was added to the plasma and TBS mixture before activation of coagulation. The plate was read every 12 seconds at 340 nm for 180 minutes at 37 ℃. Both turbidity and fibrinolysis plasma samples were run in triplicate. The parameters: lag phase, maximum turbidity (optical density, OD), time to maxOD, turbidity Vmax (OD/min), time to 15% lysis, time to 90% lysis and lysis speed were calculated from the curves (Figure 1B). To present the data from the tPA-ROTEM and turbidity lysis in a uniform way, we chose the 'MaxOD to 90% lysis time' as primary outcome for turbidity lysis time, and the 'OD/min from 15-90% lysis' as outcome for turbidity lysis speed, as these correspond to the lysis time and delta of the ROTEM, respectively

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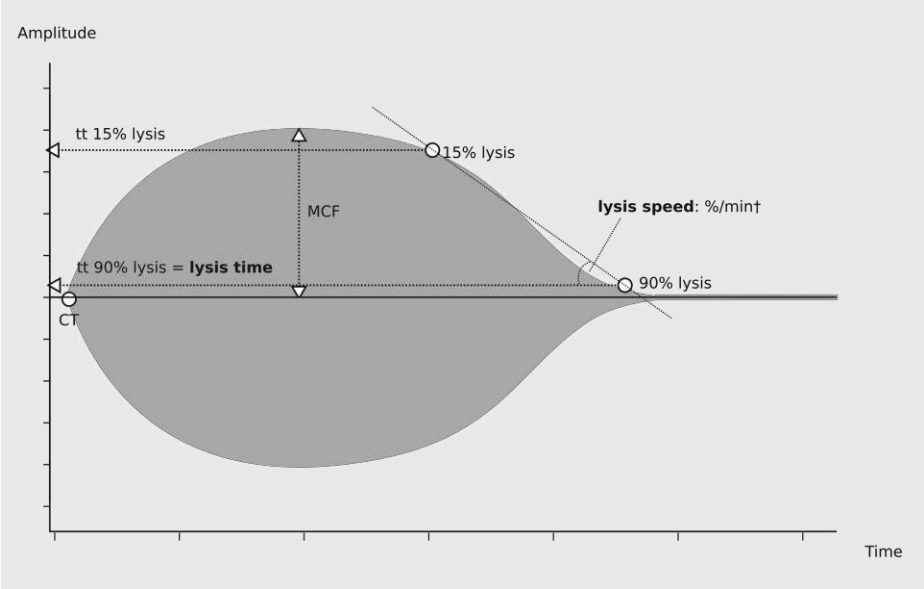
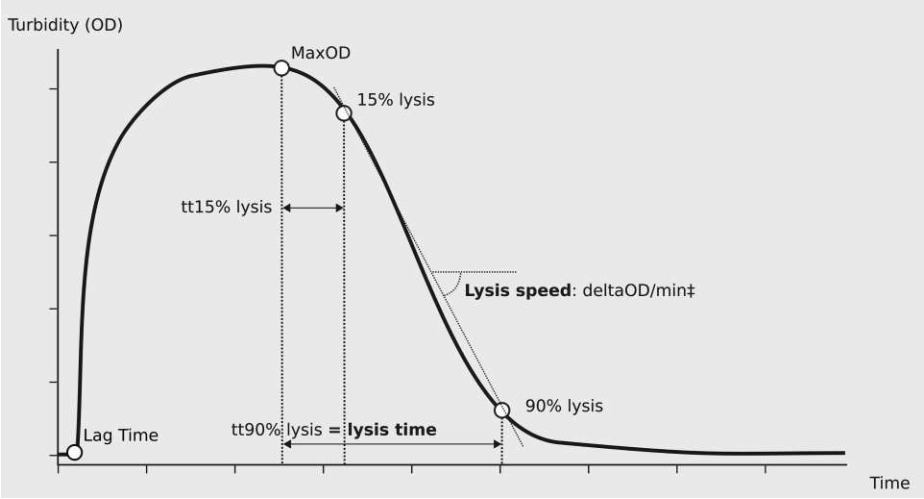
The classic euglobulin lysis time (ELT) was also performed. 7.5 ml water and a fixed amount of 130 µl 1% acetic acid was added to 500 µl of plasma. This step diluted the plasma 16.3 times. This was incubated for 10 minutes at 4℃, and then centrifuged for 10 minutes at 1780g at 4℃. The supernatant was discarded and the precipitate, containing plasminogen, fibrinogen and tPA, dissolved in 1 ml Veronal

buffer (Siemens). Clot formation was started by adding 25 µl thrombin 100 U/ml (Thrombin Reagent, Siemens). This was incubated for 5 min at 37℃. For the first 30 minutes, resolution of the clot was assessed visually every 10 minutes. Afterwards it was observed every 15 minutes for up to two hours, when the clot was almost dissolved, it was observed every 5 minutes.

Figure 1. Schematic representation of tPA-ROTEM and turbidity lysis assays



1A



1B

Important fibrinolysis parameters of the tPA-ROTEM (A) and turbidity lysis (B) are illustrated. †lysis speed (delta): %/min = 75 / (tt90% $lysis_{min}$ - tt15% $lysis_{min}$). ‡lysis speed (delta): deltaOD/min = (ΔOD between 15-90% lysis) / (tt90% $lysis_{min}$ - tt15% $lysis_{min}$). **Abbreviations**: CT: clotting time. MCF: maximal clot firmness. OD; optical density. tt; time to.

Statistical analysis

For the comparison of baseline characteristics between patients with and without reported bleeding symptoms, continuous variables were expressed as either mean with standard deviation (SD) for normally distributed traits or median with interquartile range otherwise; categorical variables were expressed as counts and percentages. Continuous variables were compared using Student's t-test for normally distributed traits; otherwise the Mann-Whitney U test was used. Categorical variables were compared using the χ^2-test or Fisher's exact test when frequencies were <5.

Multivariable linear regression was used to compare tPA-ROTEM and turbidity *lysis time* and *speed* between patients with and without bleeding symptoms, adjusted for differences in age, gender and BMI. Standardised regression coefficients represent the difference between patients with and without bleeding symptoms in SDs.

Additional multivariable linear regression models were created to identify independent determinants of the tPA-ROTEM and turbidity lysis time and speed, in *patients reporting bleeding symptoms*. For both assays fibrinogen, FII, FXI, FXII, FXIII, plasminogen, α2-AP, tPA, PAI-1 and TAFI were included as predictor variables. In the tPA-ROTEM model, the variables haemoglobin level, platelet count and leucocyte

count were also included. The outcome variables tPA-ROTEM lysis time, turbidity MaxOD to 90% lysis time, and turbidity lysis speed were log-transformed as they were non-normally distributed and thereby violated the assumptions of the linear regression models. As many variables included in the models have different measurement units or measurement ranges, we used standardised regression coefficients (Beta) to compare relative strengths of the various determinants within the models. The standardised regression coefficient of a determinant indicates the increase in standard deviations of the outcome variable when the determinant increases with one standard deviation and all other variables in the model remain unchanged. Only patients for whom values of all predictor variables and test outcomes were available were included in the multivariable regression models. Data were missing in case of a partially failed blood withdrawal, or when no blood was left to repeat the laboratory assay when it initially failed

As data were obtained from a study with another primary aim, no power calculation was performed for the current analyses. The power for the primary study aim was calculated as follows[15]: to estimate the prevalence of haemostatic abnormalities in patients reporting ≥1 bleeding symptoms, a sample size of at least 196 patients reporting bleeding symptoms was required for estimation of an expected prevalence of 15% with a maximal width of the 95%CI of ±5%. To enable comparison of the observed prevalence with patients not reporting bleeding symptoms, a random sample of these patients was taken. The inclusion rate of patients not reporting bleeding symptoms was based on maximal feasibility.

Statistical analyses were performed with IBM SPSS statistics version 24.0. Statistical significance was assumed at p-value <0.05.

Results

Baseline characteristics, haemostatic protein levels and clot formation

Data from 240 patients with and 95 patients without self-reported bleeding symptoms were available. In the group of patients with bleeding symptoms there was a greater percentage of females (72% vs. 45%, p<0.001) and mean age was higher (56 vs. 50 years, p<0.001). Furthermore, small differences in mean or median haemostatic protein levels between patients with and without reported bleeding symptoms were seen. Patients reporting bleeding symptoms had lower PAI-1 activity and higher plasminogen activity, but they also had higher FII activity and α2-AP activity levels than patients not reporting bleeding symptoms (Table 1).

Between patients with and without reported bleeding symptoms, we observed no significant differences in clot formation and strength (ROTEM extem and turbidity assay, Table S2).

Table 1. Baseline characteristics

	Patients reporting bleeding symptoms (n=240); n(%) mean(SD) or median (IQR)	Patients NOT reporting bleeding symptoms (n=95), n(%) mean (SD) or median (IQR)	p
Female	173 (72)	43 (45)	<0.001
Age, years	50 (14.8)	56 (13.4)	0.001
BMI (kg/m^2)	26.5 (5.2)	26.6 (4.0)	0.86
Haemoglobin (g/dL)			
Female	13.7 (1.1)	13.9 (1.0)	0.38
Male	15.3 (1.0)	15.0 (1.2)	0.29
Platelets (10^9/L)	270 (60)	271 (75)	0.93

Leukocytes (10^9/L)	7.1 (2.1)	7.1 (1.9)	0.83
Fibrinogen, g/L	3.2 (0.66)	3.2 (0.65)	0.81
Plasminogen, %	113.3 (19.5)	109.0 (15.0)	0.029
α2-AP, %	110.7 (12.8)	107.7 (10.1)	0.041
tPA activity, %	0.52 (0.36-0.71)	0.50 (0.34-0.67)	0.41
PAI-1 activity, %	0.16 (0.0-0.58)	0.26 (0.038-1.38)	0.018
TAFI, %	111 (91.1-120.8)	105.4 (94.4-120.8)	0.061
FII activity, %	105.9 (11.2)	102.7 (10.7)	0.017
FXI activity, %	126.5 (32.0)	132.2 (24.4)	0.32
FXII activity, %	112.2 (33.4)	113.4 (28.1)	0.76
FXIII activity, %	139.5 (26.7)	144.5 (30.3)	0.15

Abbreviations: α2-AP; α2-antiplasmin. BMI: body mass index. IQR: interquartile range. PAI-1: plasminogen activator inhibitor 1. TAFI: thrombin activatable fibrinolysis inhibitor. tPA: tissue plasminogen activator. SD: standard deviation.

Lysis capacity in patients with and without bleeding symptoms

Table 2 shows the results of the multivariable regression analyses, comparing lysis results of the tPA-ROTEM and turbidity assays between patients with and without reported bleeding symptoms. Remarkably, instead of enhanced clot lysis, patients reporting bleeding symptoms had signs of *slower* lysis speed and *longer* lysis time than those without bleeding symptoms in the tPA-ROTEM. When adjusting for age, gender and BMI, patients reporting bleeding symptoms had significantly longer tPA-ROTEM lysis time (β 0.29 (95%CI -0.042 to 0.53), p=0.022) and lower tPA-ROTEM lysis speed (delta) (β -0.35 (95%CI -0.60 to -0.10), p=0.007) than patients without bleeding symptoms, respectively. Turbidity lysis time (adjusted β 0.23 (95%CI -0.014 to 0.46), p=0.065) was not significantly longer and turbidity lysis speed (adjusted β –0.15 (95%CI -0.40 to 0.11), p=0.26) was not significantly slower in patients with vs

without reported bleeding symptoms. We performed these analyses in patients without any haemostatic abnormalities (von Willebrand, coagulation or platelet function defects), and in patients reporting menorrhagia and postoperative bleeding (as these symptoms are often seen in patients with hyperfibrinolytic defects), but results were comparable to those presented in Table 2 (see Table S3 and S4).

The classic ELT did not differ between patients with and without reported bleeding symptoms (median 105 (IQR 85-120) vs. 105 (IQR 80-120) minutes (p=0.58), respectively) (Table S2).

Table 2. Standardised regression coefficients for tPA-ROTEM and turbidity lysis results in patients with and without bleeding symptoms

Variables	Not adjusted		Adjusted†	
tPA-ROTEM: Lysis time (n=327)	**Beta (95%CI)**	**p**	**Beta (95%CI)**	**p**
Patient reporting bleeding symptoms (vs. NOT reporting bleeding symptoms)	0.23 (-0.010 to 0.47)	0.060	0.29 (0.042 to 0.53)	0.022
tPA-ROTEM: delta (n=320)	**Beta (95%CI)**	**p**	**Beta (95%CI)**	**p**
Patient reporting bleeding symptoms (vs. NOT reporting bleeding symptoms)	-0.29 (-0.53 to -0.051)	0.018	-0.35 (-0.60 to -0.10)	0.007
Turbidity Lysis: Lysis time (n=326)	**Beta (95%CI)**	**p**	**Beta (95%CI)**	**p**
Patient reporting bleeding symptoms (vs. NOT reporting bleeding symptoms)	0.12 (-0.12 to 0.37)	0.32	0.23 (-0.014 to 0.46)	0.065
Turbidity Lysis: Delta (n=326)	**Beta (95%CI)**	**p**	**Beta (95%CI)**	**p**
Patient reporting bleeding symptoms (vs. NOT reporting bleeding symptoms)	-0.064 (-0.31 to 0.18)	0.61	-0.15 (-0.40 to 0.11)	0.26

Multivariable linear regression analysis, with Beta representing the mean difference in tPA-ROTEM lysis time, tPA-ROTEM delta, turbidity lysis time from MaxOD to 90% lysis, and turbidity lysis speed (90-15% OD/min), expressed in SD, in patients reporting bleeding symptoms vs. not reporting bleeding symptoms, both unadjusted and †adjusted for sex, age and BMI. tPA-ROTEM and turbidity

lysis outcomes were log transformed. A positive Beta means that patients reporting bleeding symptoms have a higher value of the outcome than patients not reporting bleeding symptoms (expressed in SDs). Models were based on patients without any missing variables. As 7 patients had no complete lysis after 120 min in the tPA-ROTEM, tPA-ROTEM lysis time was set on 120 min but delta was not calculated (explaining the difference of 7 patients between tPA-ROTEM LT and delta).
Abbreviations: BMI: body mass index. CI: confidence interval. SD: standard deviation. tPA: tissue plasminogen activator.

Determinants of the tPA-ROTEM and turbidity lysis assay results

To identify independent determinants of both the tPA-ROTEM and the turbidity lysis results in *patients who report bleeding symptoms*, we performed multivariable linear regression analysis including fibrinogen, FII, FXI, FXII, FXIII, plasminogen, α2-AP, tPA, PAI-1 and TAFI as predictor variables. In the tPA-ROTEM model the variables haemoglobin level, platelet count and leucocyte count were also included. In these models, we focused on patients reporting bleeding symptoms, as they differed from the patients without bleeding symptoms regarding lysis time and speed.

tPA-ROTEM

Higher FII, α2-AP, TAFI and PAI-1 levels significantly prolonged lysis time. A higher plasminogen level significantly shortened the lysis time. Lysis speed was significantly decreased by a higher α2-AP and TAFI levels, while higher plasminogen and FXII activity levels significantly accelerated lysis speed (Table 3).

Table 3. Determinants of tPA-ROTEM results: lysis time and lysis speed

Patients reporting bleeding symptoms	Lysis time (n=219)		Lysis speed (delta) (n=214)	
Variables	**Beta (95%CI)**	**p**	**Beta (95%CI)**	**p**
Fibrinogen (SD)	0.11 (-0.40 to 0.26)	0.15	-0.11 (-0.27 to 0.059)	0.21
FII (SD)	0.21 (0.050 to 0.37)	0.010	-0.15 (-0.32 to 0.026)	0.095
FXI (SD)	0.10 (-0.031 to 0.24)	0.13	-0.12 (-0.27 to 0.027)	0.11
FXII (SD)	-0.10 (-0.24 to 0.030)	0.13	0.21 (0.057 to 0.35)	0.007
FXIII (SD)	0.026 (-0.11 to 0.16)	0.70	0.093 (-0.052 to 0.24)	0.21
Plasminogen (SD)	-0.18 (-0.32 to -0.041)	0.012	0.17 (0.014 to 0.32)	0.032
α2-AP (SD)	0.13 (0.001 to 0.26)	0.049	-0.22 (-0.36 to -0.075)	0.003
tPA (SD)	-0.057 (-0.20 to 0.087)	0.44	0.061 (-0.099 to 0.22)	0.45
PAI-1 (SD)	0.31 (0.15 to 0.46)	<0.001	-0.18 (-0.37 to 0.018)	0.075
TAFI (SD)	0.26 (0.12 to 0.39)	<0.001	-0.23 (-0.39 to -0.083)	0.003
Hb-level (SD)	-0.057 (-0.18 to 0.068)	0.37	-0.013 (-0.15 to 0.13)	0.86
Platelet count (SD)	0.11 (-0.040 to 0.26)	0.15	-0.091 (-0.26 to 0.074)	0.28
Leucocyte count (SD)	-0.078 (-0.22 to 0.060)	0.27	0.060 (-0.091 to 0.21)	0.43

Multivariable linear regression analysis, representing the mean change in tPA-ROTEM lysis time and delta in SD, with one SD increase in factors/age/blood cell counts in all patients. The outcome lysis time was log transformed, the variables were not. Models were based on patients without any missing variables. As 5 patients had no complete lysis after 120 min, lysis time was set on 120 min but delta was not calculated (explaining the difference of 5 patients between LT-delta). **Abbreviations**: CI: confidence interval. Hb: haemoglobin. PAI-1: plasminogen activator inhibitor 1. TAFI: thrombin activatable fibrinolysis inhibitor. tPA: tissue plasminogen activator. SD: standard deviation.

Turbidity lysis

Higher PAI-1 and TAFI levels significantly prolonged the lysis time. Lysis speed was significantly decreased by a higher α2-AP and TAFI level, while a higher fibrinogen level increased lysis speed (Table 4).

Table 4. Determinants of turbidity lysis results: lysis time and lysis speed

Patients reporting bleeding symptoms	Lysis time (n=226)		Lysis speed (n=226)	
Variables	**Beta (95%CI)**	**p**	**Beta (95%CI)**	**p**
Fibrinogen (SD)	0.16 (-0.003 to 0.32)	0.055	0.18 (0.022 to 0.34)	0.026
FII (SD)	0.11 (-0.062 to 0.29)	0.20	-0.11 (-0.28 to 0.066)	0.22
FXI (SD)	0.085 (-0.065 to 0.24)	0.27	-0.091 (-0.24 to 0.058)	0.23
FXII (SD)	-0.078 (-0.23 to 0.070)	0.30	0.088 (-0.058 to 0.24)	0.24
FXIII (SD)	0.023 (-0.13 to 0.17)	0.77	-0.025 (-0.17 to 0.12)	0.74
Plasminogen (SD)	-0.15 (0.31 to 0.012)	0.069	0.15 (-0.008 to 0.32)	0.062
α2-AP (SD)	0.13 (-0.021 to 0.28)	0.093	-0.18 (-0.33 to -0.032)	0.018
tPA (SD)	0.013 (-0.16 to 0.18)	0.088	0.044 (-0.12 to 0.21)	0.60
PAI-1 (SD)	0.18 (0.009 to 0.36)	0.039	-0.16 (-0.33 to 0.011)	0.067
TAFI (SD)	0.24 (0.081 to 0.39)	0.003	-0.23 (-0.38 to -0.073)	0.004

Multivariable linear regression analysis, representing the mean change lysis time and lysis speed in SD, with one SD increase in factors or age in all patients. The outcomes were log transformed, the variables were not. Models were based on patients without any missing variables. **Abbreviations**: CI: confidence interval. PAI-1: plasminogen activator inhibitor 1. TAFI: thrombin activatable fibrinolysis inhibitor. tPA: tissue plasminogen activator. SD: standard deviation.

Comparing determinants of the tPA-ROTEM and turbidity lysis results

Despite the differences between the laboratory principles of the tests, there were important similarities between the determinants of the test results. α2-AP, PAI-1 and TAFI appeared important lysis inhibitors in both the tPA-ROTEM and turbidity lysis

assays. A higher plasminogen level seemed to enhance fibrinolysis rate in both assays. It significantly shortened lysis time and increased lysis speed in the tPA-ROTEM, and showed the same trend in the turbidity lysis assay (p=0.069 and 0.062, respectively).

There were also a few differences. FII activity was independently associated with a longer lysis time in the tPA-ROTEM, but not in the turbidity lysis assay. A higher fibrinogen concentration accelerated lysis speed only in the turbidity lysis assay, whereas FXII accelerated lysis speed only in the tPA-ROTEM.

Discussion

Many patients with a mild bleeding phenotype remain undiagnosed.[10,11] Enhanced fibrinolytic capacity is often suggested as a possible explanation for the bleeding occurring in these patients. Reliable assessment of in vivo clot lysis is difficult with the current available clinical assays and this might explain why this has not been intensively investigated.[1,9-11] We aimed to assess whether the whole blood tPA-ROTEM and the plasma turbidity lysis assays could find evidence for faster lysis in patients with mild bleeding symptoms. In addition, we explored the potential of these assays to detect imbalances in the fibrinolytic pathway in patients with bleeding symptoms, by determining factors that influenced the tPA-ROTEM and turbidity lysis assays.

We did not find evidence for systemic hyperfibrinolytic capacity in patients reporting mild bleeding symptoms in comparison to patients not reporting bleeding

symptoms. tPA-ROTEM even suggested a *slower* clot lysis in these patients. Though this may appear counterintuitive, our results are in line with two papers assessing systemic clot lysis in mild bleeders. These studies, which included 95 patients with an undiagnosed mild bleeding tendency[18] and 97 women with menorrhagia[19], showed that patients with bleeding symptoms did not have faster lysis than controls. In these papers, turbidity lysis times in bleeders were slightly (but not significantly) longer than lysis times in controls. We have no good explanation for these findings.

Analysing the determinants of the tPA-ROTEM and turbidity lysis assays showed substantial roles for the fibrinolysis inhibitor proteins. As assumed, in both assays, the α2-AP and PAI-1 were important for clot lysis protection, as α2-AP inhibits free plasmin and PAI-1 inhibits tPA[1], while a higher plasminogen enhanced fibrinolysis. Also TAFI had a pronounced role in both assays. *In vivo* TAFI leads to the removal of plasminogen binding sites on fibrin, but this is mainly enhanced by the thrombin-thrombomodulin complex on endothelium.[20] However, it has been proposed that during clot formation, both plasmin and the significant thrombin burst are sufficient to activate TAFI without thrombomodulin (essential for the modulation of thrombus growth at sites away from the endothelium), helping to explain why we see TAFI effects in our clot lysis assays.[20,21] In addition, small amounts of thrombomodulin can be present in a soluble form, which might also contribute to TAFI activation in our samples.[22] Although plasminogen and the lysis inhibitors influenced both fibrinolysis assays in the same way (e.g. lysis acceleration or protection), we found some differences in the degree of effect on the tPA-ROTEM and turbidity lysis assay results (difference in standardised regression coefficients). Blood cells and platelets have previously been shown to play a role in fibrinolysis, for

example by protecting plasmin(ogen) from α2-AP by binding it to the cell surface.[23-25] Therefore, the presence of blood cells in the tPA-ROTEM and their absence in the turbidity lysis assays could contribute to these differences.

Some coagulation factors influenced the tPA-ROTEM and turbidity lysis assay results differently. In the tPA-ROTEM, a higher FII activity was associated with a significantly longer lysis time, whereas higher FXII activity increased lysis speed. Higher concentrations of FIIa are known to produce clots consisting of thinner, more tightly woven fibrin fibres, which are more difficult to lyse.[2,3] Endogenous FII activity did not play a role in the turbidity assay, presumably because clotting is triggered by thrombin (versus Tissue Factor in the tPA-ROTEM). The overall profibrinolytic activity of FXII in our whole blood tPA-ROTEM is of special interest, as previous studies showed that FXII can play both pro- and antifibrinolytic roles in plasma. It protects the clot from lysis by stimulating the formation of denser fibrin clots composed of thinner fibres, but it has also been shown to enhance lysis speed by converting plasminogen into plasmin.[26-28] Although the conversion of plasminogen into plasmin seemed only relevant at relatively low tPA concentrations in a plasma based assay[27], a recent paper showed that platelet polyphospate and FXII were found to co-localize on the activated platelet membrane in a fibrin-dependent manner, and under these conditions, FXIIa is a highly efficient and favourable plasminogen activator.[29] This could also explain why FXII enhanced lysis speed in the whole blood tPA-ROTEM, but not in the turbidity lysis assay. A factor that only significantly influenced the turbidity lysis assay was fibrinogen level; a higher fibrinogen level increased lysis speed. Whilst the conversion of fibrinogen into fibrin is essential for clot formation, both

fibrinogen and fibrin also catalyse their own destruction by increasing the conversion of plasminogen into plasmin.[1]

Our study had some limitations. The patients were heterogeneous regarding age, co-morbidities and co-medication, which could have influenced clot lysis results. In addition, females were overrepresented in the group of patients reporting bleeding symptoms, at least partly attributable to the inclusion of gender-specific bleeding questions in the anaesthesiology questionnaire. We aimed to partially compensate for these differences by adjusting for age and gender. Other limitations concern technical properties of the lysis tests, and thereby the extent to which the tests reflect the situation *in vivo*. The tPA-ROTEM and turbidity lysis cannot assess the contribution of endogenous plasma tPA levels, as an abundance of tPA is added to start fibrinolysis. However, the fact that the ELT was equal between these groups indicates that endogenous tPA activity was comparable between patients with and without reported bleeding symptoms. In addition, although the tPA-ROTEM is a whole blood test and includes more *in vivo* components than the turbidity lysis assay, both tests are unable to appraise the role of endothelial cells and blood flow on clot architecture and lysis.

Conclusion

The tPA-ROTEM and the turbidity lysis assay could not detect enhanced fibrinolytic capacity in patients with mild bleeding symptoms. This suggests that the bleeding symptoms reported by these patients cannot be explained by a hyperfibrinolytic state. Both the tPA-ROTEM and the turbidity lysis assay appeared sensitive to important fibrinolysis proteins such as PAI-1, α2-AP and TAFI, but these tests need to

be validated in patients with known hypo- or hyperfibrinolytic disorders to definitely establish their ability to detect a fibrinolytic imbalance.

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Acknowledgments

This research was supported by the Maastricht University Medical Centre+, the Sint Annadal Foundation Maastricht, CTMM INCOAG Maastricht, British Heart Foundation (BHF) (RG/13/3/30104 and PG/16/60/32292) and an incentive by CARIM to H.t.C.. H.t.C. is Fellow of the Gutenberg Research College, Gutenberg University, Mainz, Germany.

We wish to thank our research nurses J. Vrancken and M. Pavlovic for recruitment and care of study patients; the technicians of the haemostasis laboratory, especially R. van Oerle, for laboratory measurements (MUMC+); the anaesthesiologist dr. M. Lance for contributing to the concept of the study; the anaesthesiologist dr. R. Timmer and the employees of the outpatient anaesthesiology department for recruitment of patients (MUMC+); the haematologist dr. E. Beckers for consultation (MUMC+); the medical students M. Huisert, E. Bidar, K. Nemeth for assistance with data collection and laboratory measurements (Maastricht University).

Highlights

- Enhanced clot lysis is associated with bleeding, but assessment of lysis capacity remains difficult
- The turbidity lysis and tPA-ROTEM assays estimate fibrinolysis under more physiological conditions than clinically used assays
- Turbidity lysis and tPA-ROTEM could not detect enhanced clot lysis in patients with mild bleeding symptoms
- As both assays are sensitive to determinants of fibrinolysis, they may have potential to detect a fibrinolytic imbalance