Evaluation of thromboelastometry, thrombin generation and plasma clot lysis time in patients with bleeding of unknown cause: A prospective cohort study

Caroline S. B. Veen1 | Elise J. Huisman2 | Marjon H. Cnossen2 | Regina Kom-Gortat1 | Dingeman C. Rijken1 | Frank W. G. Leebeek1 | Moniek P. M. de Maat1 | Marieke J. H. A. Kruij1

1Department of Haematology, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands
2Department of Paediatric Haematology, Erasmus University Medical Center - Sophia Children’s Hospital, Rotterdam, The Netherlands

Correspondence
Marieke J. H. A. Kruij, Department of Haematology, Erasmus MC, University Medical Center Rotterdam, Room Na-808, PO Box 2040, 3000 CA Rotterdam, The Netherlands. Email: m.kruij@erasmusmc.nl

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Abstract

Introduction: Diagnostic evaluation of patients with a bleeding tendency remains challenging, as no disorder is identified in approximately 50% of patients. An impaired interplay of several haemostatic factors might explain bleeding phenotype in these patients.

Objective: To investigate whether global haemostasis assays are able to identify haemostatic abnormalities in patients with a bleeding tendency unexplained by current diagnostic laboratory tests.

Materials and methods: Patients of ≥12 years with a bleeding tendency were included from a tertiary outpatient clinic. Bleeding phenotype was assessed with the ISTH-BAT. Patients were classified as having bleeding of unknown cause (BUC) or a mild bleeding disorder (MBD) based on abnormalities assessed by routine haemostatic tests. Global haemostasis tests (rotational thromboelastometry (ROTEM), thrombin generation test (TG) and plasma clot lysis time (CLT)) were measured in all patients. The results were compared with 76 controls.

Results: One hundred and eighty-one patients were included, and 60% (109/181) was classified as having BUC. BUC patients demonstrated a significantly prolonged lag time in TG (median 7.7 minutes, IQR 6.7-8.7) and a significantly prolonged CLT (median 60.5 minutes, IQR 54.7-66.1) compared to controls. No differences in ROTEM variables were found. Patients with MBD showed an impaired thrombin generation with a significantly decreased ETP (median 1024 nmol/L*min, IQR 776-1355) and peak height (median 95 nmol/L, IQR 76-138), compared to BUC patients and controls.

Conclusion: No major differences were found in ROTEM and TG variables in BUC patients compared to controls. BUC patients did have a significantly prolonged clot lysis time. The underlying mechanism for this finding is unknown.

Keywords
bleeding, bleeding of unknown cause, fibrinolysis, haemostasis, mild bleeding disorder, thrombin generation, thromboelastometry
Patients with a mild bleeding disorder present with varying symptoms, such as easy bruising, mucocutaneous bleeding, and bleeding after surgery or tooth extraction. However, in the general population bleeding symptoms are reported in more than 20% of healthy individuals. Therefore, diagnostic evaluation of patients with a bleeding disorder is a challenging process. Application of routine diagnostic laboratory tests in patients with clinically relevant bleeding leaves around 50% of patients without a diagnosis. Subsequently, these patients are classified as patients with bleeding of unknown cause (BUC). Patients with a clinically relevant bleeding phenotype are also regularly diagnosed with mild haemostatic defects which may not sufficiently explain the patient’s bleeding phenotype (mild bleeding disorders, MBD). As a clear diagnosis is lacking in these patients, the most appropriate treatment regimen also remains uncertain.

An impaired interplay between several mild haemostatic defects may explain bleeding phenotype in this patient category. Global haemostatic assays may increase insight into the pathogenesis of BUC, as other components of the haemostatic system on blood coagulation are investigated more thoroughly. Rotational thromboelastometry (ROTEM) provides a graphical representation of blood clot formation and fibrinolysis, which includes contributions of erythrocytes, leucocytes and platelets. Measurement of thrombin generation (TG) has also been proposed as a promising approach to globally estimate an individual’s coagulation potential and to predict a hypercoagulable state. In addition, investigation of fibrinolysis is often omitted in the routine work-up of patients with a bleeding tendency. It is however known that clots made from the plasma of haemophilia patient show altered characteristics and higher susceptibility to fibrinolysis.

In order to gain more insight into the pathophysiological mechanisms of bleeding symptoms in patients with BUC and MBD, and to investigate the diagnostic value of global haemostasis tests in these patients, we investigated the role of these global tests in the diagnostic work-up.

2 | MATERIALS AND METHODS

2.1 | Study population

Patients, aged 12 years or older, referred to the outpatient haematology and paediatric haematology clinics of our tertiary clinics, the Erasmus University Medical Center and Sophia Children’s Hospital, for haemostatic screening between 1 June 2016 and 1 March 2018 due to a clinically relevant bleeding tendency were prospectively included. Patients previously diagnosed with a bleeding disorder or diagnosed with an established bleeding disorder after a first laboratory panel (eg von Willebrand’s disease, haemophilia or platelet disorder), patients using anticoagulant, antiplatelet or nonsteroidal anti-inflammatory drugs, pregnant women and women <3 months postpartum were not eligible for study inclusion. A total of 76 sex-matched healthy individuals were included as control group. These healthy individuals were recruited among employees and students of the Erasmus MC University Medical Center. This study was subject to the Medical Research Involving Human Subjects Act and approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2016-218). Written informed consent was obtained from each participant.

2.2 | Medical bleeding history and bleeding assessment tool

Upon inclusion, all surgical interventions, tooth extractions, obstetric history and detailed family history were documented. A bleeding score (BS), based on the history of bleeding events, was calculated by the ISTH-Bleeding Assessment Tool (ISTH-BAT), with cut-off values ≥4 in males, ≥6 in females and ≥3 in children. Laboratory tests were performed in a stepwise manner. The first step included a full blood count, ABO blood type, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration according to von Clauss, determination of VWF antigen (VWF:Ag), activity (VWF:GPIbM) and collagen binding (VWF:CB), one-stage assay FVIII:C and FIX:C and VWF-multimer analysis in case of low VWF. Platelet function was assessed with the collagen-epinephrine and collagen-ADP cartridges on the platelet function analyzer (PFA-200). As second step, FVII:C, FXI:C and FXIII:C, α2-antiplasmin, and light transmission aggregometry (LTA) were performed. Measurements of VWF:Ag/Act/CB and FVIII:C were repeated at least once.

Blood sampling was performed using the Vacutainer system (Becton Dickinson) containing sodium citrate (final concentration 0.109 mol/L) or EDTA (1.8 mg/mL, Plymouth). Citrated blood was centrifuged at 2000 g for 10 minutes at room temperature, followed by 14 000 g for 10 minutes centrifugation of plasma at room temperature. Platelet poor plasma (PPP) samples were stored in aliquots at −80°C until analysis, when indicated. Routine coagulation tests aPTT (Actin FS), PT (Thromborel S) and fibrinogen (Thrombin Reagent) were measured on a Sysmex CS-5100 (Siemens Healthcare Diagnostics BV). Collagen-ADP (C-ADP) and collagen-epinephrine (C-Epi) cartridges were used to measure closure times (CT, seconds) on the PFA-200 (Siemens). Light transmission aggregometry (LTA) was performed on a Chrono-Log aggregometer 490 (Stago Benelux BV). VWF:Ag levels and VWF:CB activity were determined with an in-house ELISA assay. VWF activity (VWF:GPIbM) was determined with the INNOVANCE VWF Ac assay (Siemens) on a Sysmex CS-5100. FVIII:C and FIX:C were measured using one-stage clotting assays and derived from the prolongation of the clotting time (aPTT) measured on the Sysmex CS-5100 (Siemens). FXIII activity was measured using the Berichrom® FXIII kit.
Thrombinoscope BV), which consists of a mixture of tissue factor
PPP was diluted in buffer (25 mmol/L Hepes, 137 mmol/L NaCl, 3.5 mmol/L KCl, 1% (w/v) BSA, pH 7.4). The diluted plasma was
data were measured in duplicate. Alpha 2-antiplasmin level was measured using a chromogenic assay (Stachrom, Stago) on the
Sysmex CS-5100 (Siemens).

2.4 | Rotational thromboelastometry

Viscoelastic clotting measurements were performed with ROTEM®
Delta (Tem International xGmbH) tests according to the manufacturer’s protocol. All investigations were performed within 2 hours
after blood collection and the assays ran for 60 minutes. Extrinsic and intrinsic coagulation was measured with the EXTEM and the
INTEM assays. The influence of fibrinogen on clot firmness was estimated with the platelet-inactivated FIBTEM assay. The following
ROTEM parameters were analysed: clotting time (CT, sec); clot formation time (CFT, sec); maximum clot firmness (MCF, mm); and
maximal lysis (ML, %).

2.5 | Thrombin generation

Thrombin generation was assessed using the calibrated auto-
mated thrombogram (CAT) assay (Diagnostica Stago) in accordance
with the manufacturers’ instructions, as described previously. Briefly, PPP was added to PPP Reagent 1 PM TF (PPP-Reagent LOW,
Thrombinscope BV), which consists of a mixture of tissue factor
(TF: 1 PM final concentration in plasma) and phospholipids. Plasma
each of subject was analysed in duplicate. Acquisition of thrombin generation parameters was performed using the Thrombinscope
software (Diagnostica Stago; CAT). Four parameters were derived from the thrombin generation curve: lag time (min), time to peak (tt-
peak, min), endogenous thrombin potential (ETP, nmol/L*min) and
peak height (nmol/L).

2.6 | Plasma clot lysis assay

The plasma clot lysis assay was performed as described before.17,18
PPP was diluted in buffer (25 mmol/L Hepes, 137 mmol/L NaCl,
3.5 mmol/L KCl, 1% (w/v) BSA, pH 7.4). The diluted plasma was
added to a reaction mixture, containing tissue factor (TF, Innovin,
1000 times diluted; Dade Behring), CaCl₂ (17 mmol/L), TPA
(30 ng/mL, Actilyse, Boehringer Ingelheim), phospholipid vesicles
(10 µmol/L, Rossix Mölndal) and potato carboxypeptidase inhibi-
tor (PCI, an inhibitor of activated TAFI) (30 µg/mL) when indicated.
The concentrations refer to the final concentrations in the clot.
In a microplate reader (Victor™, PerkinElmer), the optical density
at 405 nm was measured every minute for 300 minutes at 37°C.
The clot lysis time (CLT) was the time from midpoint of minimum
clot formation to maximum turbidity, which represents clot formation,
to the midpoint of maximum turbidity to minimum turbidity, which
represents clot lysis. CLTs with and without the addition of PCI
were measured in duplicate.

2.7 | Reference ranges

Reference ranges for ROTEM, thrombin generation and plasma
clot lysis time are based on 76 healthy controls, calculated with the
Reference Value Advisor software (v2.1) which closely follows the
CLSI guideline.19,20

2.8 | Definition of diagnoses

A MBD was defined as the presence of a hereditary bleeding dis-
order, specified as follows: low VWF—VWF activity levels between
0.30-0.50 U/mL and ratio of FVIII:C to VWF:Ag > 0.6;21 PFD: ab-
normalities found using light transmission aggregation testing (LTA),
not fitting the pattern of any known platelet function disorder;22
and isolated coagulation factor deficiency: deficiency of a coagula-
tion factor, other than FVIII (haemophilia A) or FIX (haemophilia
B), with laboratory criteria as proposed by the European Network
of Rare Bleeding Disorders.6,23 Bleeding was considered as bleed-
ing of unknown cause (BUC) based on the absence of haemostatic
abnormalities after extensive laboratory investigation, as described
before.1,7,24

2.9 | Statistics

We used descriptive statistics to summarize baseline character-
istic of the study population. In case of a skewed distribution,
data are presented as median and interquartile range (IQR), and
compared using an independent sample test. Categorical data are presented as numbers with percentages and compared
using a Pearson Chi-square test. In multiple logistic regression,
models were adjusted for age, sex, BMI, platelet count, fibrinogen,
VWF, FVIII:C and FXIII:C as appropriate. Outcomes are reported
as odds ratios (ORs) followed by the 95% confidence interval (CI).
Multiplicty correction was not performed because of the hypo-
thesis-generating approach of the study. A P-value of <.05 was con-
sidered statistically significant. All analyses were performed with
SPSS version 24.0 (IBM, Armonk, NY, USA).

3 | RESULTS

One hundred and eighty-one patients were referred to our hospital
with a clinically relevant bleeding tendency and eligible for inclusion,
and 76 healthy individuals were included as healthy controls. The
majority of study participants was female (84% of patients and 86%
of healthy controls). Mean age was 33.6 years (SD 17.3) for patients,
with 53/181 (29%) adolescent patients ≥ 12 years, and 35.8 years
(SD 12.3) for healthy controls, see Table 1. For study protocol and
flow of inclusion see Figure 1.
A total of 120/181 (66%) patients were classified as having BUC. Sixty patients were classified as having a MBD, with platelet function disorders (43%) and low VWF (35%) being most prevalent. BUC patients consisted of a higher percentage of adults (80% versus 52% in MBD, \(P < .01\)) and had a higher median age (33 year, IQR 24-50 year) than MBD patients (20 year, IQR 15-39 year, \(P < .01\)). Significantly less BUC patients had blood type O (40%) than MBD patients (53%, \(P < .05\)). BUC patients had a median BS of 5 (IQR 3-8), compared to a median BS of 7 (IQR 6-9) in patients with MBD (\(P < .01\)), with only 54% of BUC patients presenting with an abnormal BS, compared to 88% of MBD patients (\(P < .01\)) (see Table 1).

As expected, patients with MBD had significantly lower levels of VWF:Ag, VWF:GPIbM, VWF:CB and FVIII:C than BUC patients. Also, MBD patients had a significantly lower platelet count and increased aPTT. No differences in haemostatic variables were found between BUC patients and healthy controls (see Table 2 and Figure 2). When excluding patients with a normal bleeding score, significantly lower levels of VWF:Ag, VWF:GPIbM, VWF:CB, FVIII:C and FIX:C-level were found in MBD patients compared to BUC patients (see Table 2).

When comparing BUC patients and healthy controls, no statistically significant differences were observed in thromboelastometry variables (see Figure 2 and Table S1). When adjusting for age, sex, BMI, platelet count, fibrinogen, VWF:GPIbM and FVIII:C levels by means of logistic regression analysis, no significant differences were found between BUC patients and healthy controls (Table S2). Comparing BUC patients with MBD patients, BUC patients had a significantly decreased clot formation time (CFT) in the EXTEM and INTEM assay, and a significantly increased maximum clot firmness (MCF) in the EXTEM, INTEM and FIBTEM assay (see Figure 2 and Table S1).

BUC patients had a significantly longer lag time (median 7.7 minutes, IQR: 6.6-8.7 minutes) compared to healthy controls (median 6.9 minutes, IQR: 6.0-8.6 minutes, \(P < .05\)). Other thrombin generation parameters were not different between BUC patients and healthy controls (Table S1). When adjusting for age, sex, BMI, platelet count, fibrinogen and FXIII:C levels by means of logistic regression analysis, also no significant differences were found between BUC patients and healthy controls (Table S2). In MBD patients, impaired thrombin generation was found, with a significantly decreased ETP and peak height compared to BUC patients (see Figure 2) and healthy controls.
Remarkably, a significant longer CLT in BUC patients (PCI - median 60.3 minutes, IQR 54.7-66.0 minutes and PCI + 41.3 minutes, IQR 38.0-46.2 minutes) was found compared to healthy controls (PCI- median 57.4 minutes, IQR 53.9-61.7 minutes and PCI + 38.9, IQR 36.3-42.5 minutes, \( P = .03 \) and \( P < .01 \) respectively) (Table S1). However, when adjusting for age, sex, BMI, platelet count, fibrinogen and FXIII:C levels by means of logistic regression analysis, no significant differences were found between BUC patients and healthy controls (Table S2). Overall, no differences were found in CLT between BUC patients and MBD patients, both with and without adjustment for age, sex, BMI, platelet count, fibrinogen and FXIII:C levels.

In thromboelastometry, thrombin generation and clot lysis time variables, no additional significant differences were found between BUC and MBD patients, after excluding patients with a normal bleeding score (see Table 2 and Table S1).

The ETP was significantly lower in patients with an abnormal bleeding score (median 1223 nmol/L*min, IQR: 923-1516 nmol/L*min) compared to patients with a normal bleeding score (median 1055 nmol/L*min, IQR: 828-1363 nmol/L*min, \( P = .046 \)). Furthermore, patients with an abnormal bleeding score had a significantly longer CT (median 65 minutes, IQR: 58-72 minutes) in the EXTEN assay and significantly lower MCF (median 16 mm, IQR: 13-19 mm) in the FIBTEM assay compared to patients with a normal score (CT-EXTEM: median 67 minutes, IQR: 63-73 minutes, \( P = .048 \) and MCF-FIBTEM: median 15 mm, IQR: 11-18 mm, \( P = .04 \)). Plasma clot lysis time was comparable in patients with an abnormal and normal bleeding score (see Figure 3 and Table S4).

**DISCUSSION**

This study reports on a cohort of 181 patients referred for analysis of a bleeding tendency in whom no major bleeding disorder was diagnosed. After routine haemostatic testing, 66% of patients remained undiagnosed and were classified as having bleeding of unknown cause (BUC). The other 34% of patients were diagnosed with a mild bleeding disorder (MBD).

We found that rotational thromboelastometry variables are within reference ranges in BUC patients and do not differ from healthy controls and MBD patients. Our results are in line with those recently described by Wieland Greguare-Sander et al\(^2\) and support their conclusion that there is no support for the additive value of rotational thromboelastometry for screening and diagnosing patients with a
(mild) bleeding tendency. Thrombin generation has been applied regularly to investigate bleeding risk in patients with a bleeding disorder. In this study, besides a significant longer lag time in BUC patients, thrombin generation parameters did not differ between patients with BUC and healthy controls, as also shown in previous studies. This finding was, however, in contrast with recently published data, in which all the TG variables in BUC patients were found to be significantly different from healthy controls. Patients with MBDs did show a significantly impaired thrombin generation, with a decreased endogenous thrombin potential (ETP) and peak height. This finding is remarkable, however, as the used thrombin generation is a reflection of secondary haemostasis, and most patients in the MBD group are diagnosed with a disorder of primary haemostasis. Also, no evidence was found supporting a systemic hyperfibrinolytic capacity in BUC patients. In contrast, we found that clot lysis time was significantly prolonged in BUC patients compared to healthy controls, in line with 

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<th>TABLE 2</th>
<th>Haemostatic variables in different patient groups and healthy controls</th>
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<td></td>
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<td>59⁴</td>
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<td>APTT, sec</td>
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<td>Fibrinogen, g/L</td>
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<td>FXIII:C, U/mL</td>
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<td>Alpha-2-antiplasmin, U/mL</td>
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<td>53⁴</td>
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<td>Note: Data are shown as median and interquartile range [25th-75th percentile].</td>
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<td>Abbreviations: aPTT: activated partial thromboplastin time; BUC: bleeding of unknown cause; FIX:C: factor IX activity; FVII:C factor VII activity; FVIII:C: factor VIII activity; FXI:C: factor XI activity; FXIII:C: factor XII activity; HC: healthy controls; MBD: mild bleeding disorder; n.a.: not applicable; n.s.: nonsignificant; PFA: platelet function analyzer; PT: prothrombin time; VWF:Act: von Willebrand factor activity; VWF:Ag: von Willebrand factor antigen; VWF:CB: von Willebrand factor collagen binding.</td>
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<td>⁴Based on available data.</td>
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<td>⁵No of patients with abnormal bleeding score.</td>
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<td>⁶Comparison of BUC patients and healthy controls.</td>
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<td>⁷Comparison of BUC patients and patients with a MBD.</td>
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previously published data, hereby carefully rejecting hyperfibrinolysis as underlying pathophysiological mechanism for BUC. Several studies have shown that between 47% and 69% of patients will remain undiagnosed after extensive and repeated laboratory testing. When no laboratory abnormalities are found, the medical history and a bleeding score are important tools for physicians. However, the ISTH-BAT has shown to only have a limited role, as a normal score was present in 44% of BUC patients and in 21% of MBD patients. Therefore, a BAT should only serve as one of the many diagnostic tools available in the diagnostic work-up of these patients.

We confirm one of the main findings by Gebhart et al, namely, that the majority of patients being referred for bleeding symptoms is female (>80%) and that more women than men are categorized with BUC, hereby possible affirming that there is a sex-related difference in BUC rate. Women have a higher chance of manifest bleeding due to menstrual cycle and women's ability for childbirth. Other mechanisms however, such as the influence of female hormones on skin and muscle possibly leading to easy bruising, are still largely unknown. We also showed that patients with BUC were significantly older than patients with a diagnosed MBD. It has been shown that several haemostatic factors increase with age. This may explain that no abnormalities were found in this ‘older’ subgroup at time of analysis. Also, the role of comorbidities can be more pronounced in an older population, for example the influence of age and comorbidities on skin and vessels, possibly causing easy bruising or perioperative bleeding. To our knowledge, this is also one of the first studies to report BUC in adolescents, with a higher percentage of adolescents being diagnosed with a MBD than adults.

Our study has some limitations. First, one cannot exclude a possible referral bias for adolescents, with investigations possible being delayed or abandoned if the bleeding score was not very high. This might explain the increased rate of adolescents as well as the higher bleeding scores in the MBD patient group. We performed LTA for investigation of platelet function disorders. An influence of comediations on platelet function cannot be ruled out completely. For example, we did not exclude patients using selective serotonin reuptake inhibitors (SSRIs), which are shown to reduce platelet function. Due to the circadian rhythm of plasminogen activator inhibitor-1 (PAI-1), which inhibits
fibrinolysis and increases in the morning, we attempted to collect blood for plasma clot lysis assay in the afternoon. Unfortunately, this was not always possible due to logistic reasons.

Additional studies on patients without a clear diagnosis are required. In the near future, advanced techniques such as next-generation sequencing (NGS)-based gene panels or whole-exome sequencing (WES) may lead to discoveries of novel haemostatic modifiers. However, translating these results will provide a next challenge due to multi-interpretable and uncomprehensive findings such as variants of unknown significance (VUS).

5 | CONCLUSION

No major differences were found in thromboelastometry variables and thrombin generation in patients with bleeding of unknown cause (BUC), compared to healthy controls. BUC patients did have a significantly prolonged clot lysis time, possibly indicating an impaired or decreased fibrinolysis. In MBD patients, an impaired thrombin generation was found. At this point, however, we do not recommend implementation of thromboelastometry, measurement of thrombin generation and measurement of plasma clot lysis time in the diagnostic process of patients with bleeding of unknown cause.

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ORCID
Caroline S. B. Veen https://orcid.org/0000-0002-7671-9308
Frank W. G. Leebeek https://orcid.org/0000-0001-5677-1371
Marieke J. H. A. Kruip https://orcid.org/0000-0002-0265-4871

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