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ORIGINAL ARTICLE

Thrombin-generating potential, plasma clot formation, and clot lysis are impaired in patients with bleeding of unknown cause

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Abstract

Background: In a large proportion of patients with a mild to moderate bleeding tendency no diagnosis can be established (bleeding of unknown cause, BUC).

Objectives: To investigate possible dysfunctions in thrombin generation and plasma clot formation and lysis in patients with BUC from the Vienna Bleeding Biobank (VIBB).

Patients and Methods: Thrombin generation and plasma clot properties of 382 BUC patients were compared to those of 100 healthy controls and 16 patients with factor VIII (FVIII) activity ≤50%.

Results: Thrombin generation was significantly impaired in BUC patients compared to healthy controls, exhibiting a prolonged lag time and time to peak and decreased maximum thrombin generation, velocity index, and area under the curve (AUC). The assessment of clot formation and lysis in BUC patients revealed a lower clot formation rate (Vmax), resulting in a longer TTP, increased absorbance (ΔAbs), and a shorter clot lysis time (CLT) than in healthy controls. Comparing patients with FVIII activity ≤ 50% to those with BUC, parameters of thrombin generation and clot formation and lysis were either stronger or comparably impaired. Bleeding severity did not correlate with parameters of thrombin generation, clot formation, or clot lysis.

Conclusion: Patients with BUC have an impaired hemostatic capacity reflected by a lower thrombin-generation potential, a lower clot formation rate, increased clot turbidity, and shorter clot lysis time, which might contribute to their increased bleeding tendency. Assays monitoring these parameters can alert physicians of hemostatic impairment and should be considered in situations where traditional hemostatic lab tests fail to reveal the clinical bleeding tendency.

KEYWORDS
bleeding, blood coagulation, clot lysis time, hematologic test, hemostasis

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1 | INTRODUCTION

Mild to moderate bleeding tendency is a frequent cause for detailed investigations of the plasmatic coagulation system and platelet function. However, in the majority of these patients no underlying bleeding disorder can be identified, leading to the categorization as patients with bleeding of unknown cause (BUC).[1–3] Although the bleeding phenotype and severity are not different from patients with a diagnosis of a specific bleeding disorder,[1] the mechanisms underlying the bleeding tendency are unknown in these patients.

Available routine coagulation tests, such as activated partial thromboplastin time (aPTT) and prothrombin time (PT), do not distinguish patients with BUC from healthy controls,[1,2] perhaps because the endpoint of measurement is the formation of a fibrin clot, which occurs when only 5% of thrombin is formed.[4] Therefore, a global test that evaluates both formation and decay of thrombin may better reflect the hemostatic potential of BUC patients.[5] Decreased generation of thrombin has been shown in patients with known coagulation disorders such as von Willebrand disease (VWD) and hemophilia.[6–9] Although a previous small study by our group did not reveal disturbed thrombin generation in BUC patients compared to healthy controls,[10] data from a large cohort of patients with BUC are not available.

Analysis of clot formation, structure, and resistance to fibrinolysis is another method used to investigate hemostatic capacity. Altered clot characteristics have been reported in both thrombotic [11–14] and bleeding disorders.[9,15–17] In patients with hemophilia A and B, clot formation is delayed and results in looser clots with thicker fibrin fibers.[9,15–17] In a small BUC patient cohort, our group demonstrated that in the presence of tissue plasminogen activator, the plasma clot formation rate is reduced in BUC patients compared to healthy controls.[18]

The present study investigated the thrombin generation potential and clot formation and lysis in a large, well-defined cohort of patients with BUC from the VIBB. We aimed to determine whether these global assays reflect the hypocoagulable state of these patients.

Essentials

- Many patients with a bleeding tendency lack a diagnosis (BUC).
- We compared thrombin generation, clot formation, and lysis of BUC patients and healthy controls.
- Thrombin generation and clot formation and lysis were significantly impaired in BUC patients.
- Bleeding severity did not correlate with thrombin generation or plasma clot properties.

2 | PATIENTS AND METHODS

2.1 | Selection of patients with BUC and patients with factor VIII levels below 50%

Between October 2009 and November 2017, 547 adult patients with a mild to moderate bleeding tendency were recruited in the VIBB.[1] This ongoing single-center study included all patients ≥4 years who were referred to the hemostasis outpatient department of the Medical University of Vienna for the investigation of a bleeding disorder. Patients with a previously diagnosed defect of plasma coagulation or platelet function were not included in the VIBB. Further exclusion criteria were surgery or delivery within the last 6 weeks, bacterial infection within the last 2 weeks, and acute phase reaction at inclusion. Patients with active malignancy, pregnancy, intake of anticoagulants/antiplatelet/anti-inflammatory drugs (last 5 to 10 days), and thrombocytopenia (<100 × 10^9/L) were also not included. All participants gave written informed consent before study inclusion. Routinely, a blood count, differential blood count, renal and liver function, and parameters of inflammation and iron deficiency were assessed in each patient. The detailed coagulation tests performed in every patient are listed in Table S1. Each patient's personal medical and bleeding history was recorded by trained personnel at study inclusion. Bleeding severity was assessed using a standardized bleeding score (Vicenza bleeding score).[19] From June 2013, we also recorded the ISTH-BAT bleeding score, which is available for 186 patients (34%). The Vicenza bleeding score, which correlated strongly with the ISTH-BAT bleeding score (R = 0.819, P value <0.001), was used for further analysis.[20] All procedures were conducted in accordance with the Declaration of Helsinki of 1975 and the study was approved by the Ethics Committee of the Medical University of Vienna (EC No 603/2009).

Of the 547 VIBB patients, 149 were not included in the current analysis. Patient selection is depicted in the STROBE diagram in Figure 1. We excluded patients with repeatedly abnormal results in the assessment of platelet function by light transmission aggregometry with two or more agonists (definite platelet function disorder, n = 34) and patients with abnormal results in light transmission aggregometry in whom only one assessment was available or with repeated abnormalities not meeting the criteria of a definite PFD (possible PFD, n = 58) from the current analysis. Furthermore, patients with VWD (VWF:Ag or VWF:RCo levels < 30 IU/dL, n = 9) or low VWF (VWF:Ag or VWF:RCo levels 30-50 IU/dL, n = 31) with FVIII > 50%, patients with FIX ≤ 50% (n = 5), FXI ≤ 60% (n = 3), FXIII ≤ 10% (n = 1), and three patients with hypofibrinogenemia/dysfibrinogenemia were not included. None of our patients had alpha2-antiplasmin deficiency (<70%).[21,22] In 382 patients no underlying cause for the bleeding tendency was identified, as all coagulation and platelet function tests revealed normal results. These patients were categorized as BUC patients and are included in the current study.

In addition, we included 16 patients with factor VIII (FVIII) activity levels ≤ 50% from the VIBB for comparison. Of these, 8 patients had mild (FVIII activity: 21%-38%) and 1 patient had moderate

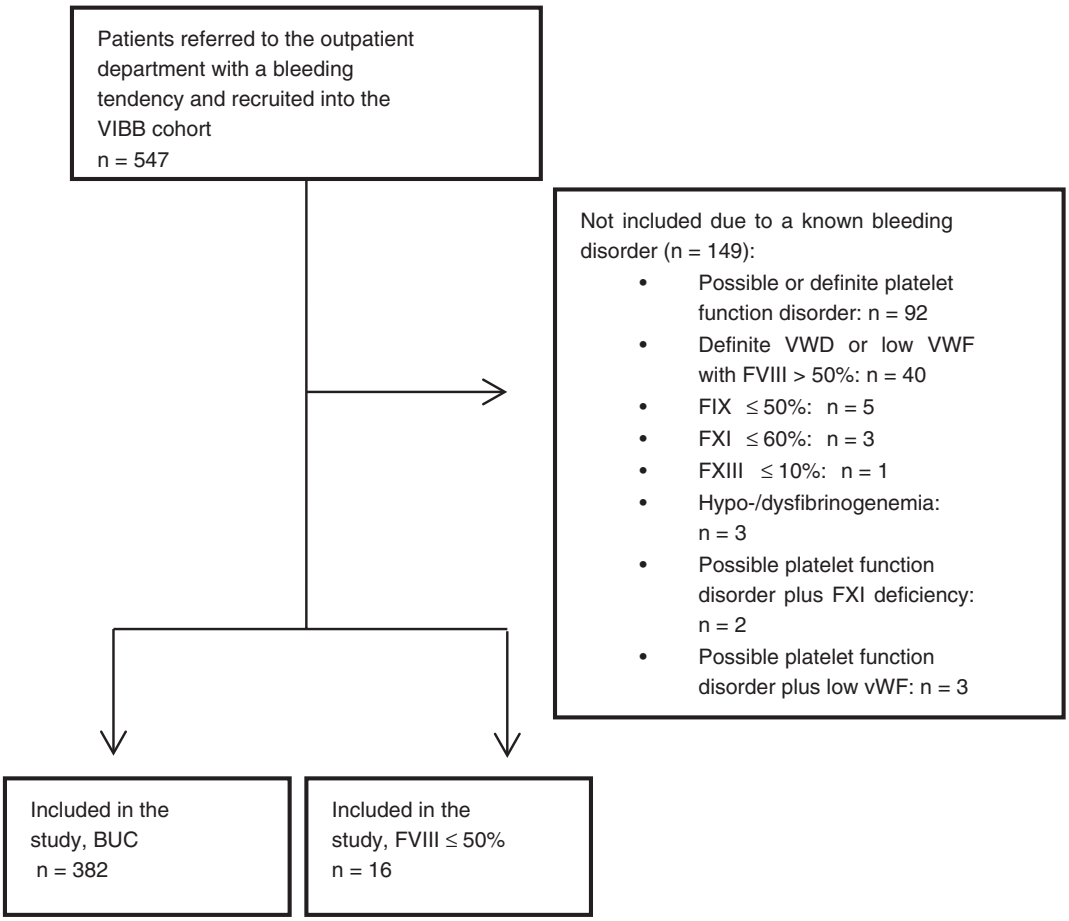

FIGURE 1 Patients included in this study. BUC, bleeding of unknown cause; FIX, factor IX activity; FVIII, factor VIII activity; FXI, factor XI activity; FXIII, factor XIII activity; VWD, von Willebrand disease

hemophilia A (FVIII activity 3%). Three patients were diagnosed with VWD (type 1: *n* = 2, type 2 Normandy: *n* = 1), and 4 had low VWF. We used patients with FVIII < 50% as a comparison, as FVIII deficiency is a well-established cause of a bleeding tendency. Furthermore, studies of FVIII-deficient patients have shown impaired (delayed and/or decreased) thrombin generation, as well as altered clot formation and lysis,[7–9,15–17] and we previously used FVIII-deficient patients to validate findings in a smaller study of patients with BUC.[18] One hundred sex-matched and age-matched unrelated healthy controls with a negative personal and family bleeding history were recruited for comparison.

2.2 | Blood sampling

For performance of the thrombin generation assay and the clot formation and lysis assay, venous blood was drawn by venipuncture using a 21-gauge butterfly needle (0.8 × 19 mm; Greiner Bio-One, Kremsmünster, Austria) into a trisodium citrate vacuette tube (Greiner Bio-One, Kremsmünster, Austria), centrifuged at 2500***g*** for 15 min at 15°C to obtain platelet-poor plasma and then stored at <−70°C at the MedUni Wien Biobank (www.biobank.at) until testing. MedUni Wien Biobank's preanalytical protocols have been published previously.[23]

2.3 | Thrombin generation assay

For the determination of thrombin generation we used a commercially available kit (Technothrombin, Technoclone, Vienna, Austria), which measures the fluorescence resulting from cleavage of a fluorogenic substrate by thrombin. We used the low concentration of phospholipid micelles containing recombinant human tissue factor to activate the coagulation cascade. According to the manufacturer's information, the approximate recombinant tissue factor concentration in the reaction mixture is <0.3 pmol/L. The following parameters were analyzed using a specifically adapted software (Technothrombin TGA, Vienna, Austria): lag time (time that is required for thrombin burst, minutes), maximum thrombin generation (peak, nmol/L), TTP (velocity of thrombin generation, minutes), velocity index (compound index including lag time and time to peak; peak/(time to peak − lag time), nmol/L/min), AUC (nmol/L × min).

2.4 | Plasma clot formation and lysis assay

Plasma clot formation and lysis were turbidimetrically assessed using the method published by Wolberg et al[16,24] The assays for clot formation and clot lysis were combined, as recommended by the SSC of the ISTH.[25] Briefly, calcium chloride (20 mM, final),

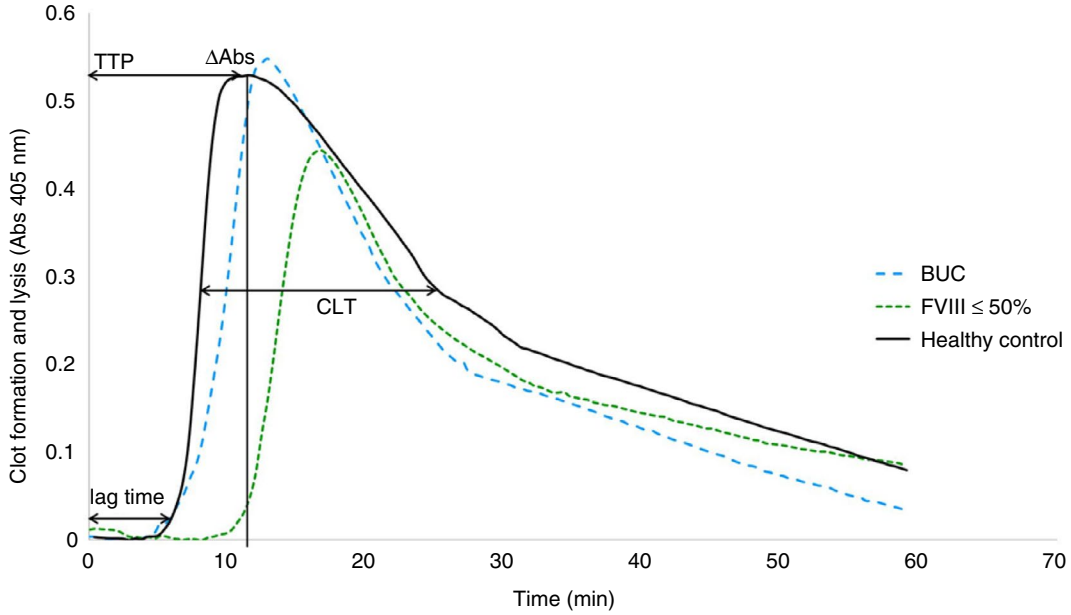

FIGURE 2 Representative curves of plasma clot formation and lysis from one patient with BUC, one patient with FVIII ≤50%, and one healthy control. ΔAbs, maximum absorbance at plateau; BUC, bleeding of unknown cause; CLT, clot lysis time; FVIII, factor VIII activity; TTP, time to peak

phospholipids (4 μmol/L, final), tissue factor (Innovin, 2 pmol/L final), and tissue plasminogen activator (333 ng/mL final) were added to citrated plasma. Turbidity was monitored by absorbance (optical density, OD) on a Thermo Scientific microplate reader and the following parameters were analyzed using the ScanIT software (Figure 2): lag time (time that is required for clot formation, minutes), maximum absorbance at plateau (ΔAbs, OD), TTP (time to maximum absorbance at plateau, minutes), maximum rate of turbidity increase (Vmax, OD/min), and CLT (time from 50% of peak OD during clot formation to 50% decrease in turbidity from peak OD, minutes).

2.5 | Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS IBM Version 24.0). Continuous variables are expressed as median (25th-75th percentile) unless stated otherwise. Continuous variables that were not normally distributed were log-transformed prior to statistical analysis. Unadjusted group comparisons were performed using the Mann-Whitney *U* test and the chi-square test was used to compare categorical variables. Analyses of covariance models were performed to evaluate adjusted group comparisons of the thrombin generation and the clot formation and lysis parameters. Correlations of metric variables were calculated using the bivariate Spearman-rho test. Multiplicity correction was not performed because of the hypothesis-generating approach of the study. The variables fibrinogen, FXIII, and body mass index (BMI) were used for adjustment comparing BUC patients and healthy controls. In case of comparison of BUC patients to FVIII patients, sex was used for adjustment. A *P* value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Clinical and laboratory characteristics of patients with BUC, healthy controls, and patients with FVIII deficiency

Clinical and laboratory characteristics of BUC patients in comparison to healthy controls and patients with FVIII ≤50% are shown in Table 1. The majority of BUC patients were female (87%). Blood group O was the predominant blood type in both BUC patients and healthy controls. The BUC patients had a slightly but significantly higher body mass index compared to healthy controls. In the laboratory results, the prothrombin time, given in percentage of normal, was significantly lower; fibrinogen was significantly higher and factor XIII activity levels were lower in BUC patients than in healthy controls, whereas there was no significant difference compared to patients with FVIII ≤50%.

As expected, in patients with FVIII ≤50%, aPTT was significantly longer and levels of VWF:Ag, VWF:RCo, and FVIII activity were significantly lower than in BUC patients and healthy controls. Interestingly, there was no difference in the bleeding score between patients with BUC and those with FVIII ≤50%.

3.2 | Thrombin generation in patients with BUC compared to healthy controls and patients with FVIII deficiency

Thrombin generation was significantly impaired in patients with BUC compared to healthy controls (Table 2, Figure S1) and, as expected, in FVIII-deficient patients compared to healthy controls (Table S2). In the BUC patient group, the lag time was prolonged,

TABLE 1 Demographic and laboratory data of patients with BUC (*n* = 382) compared to healthy controls (*n* = 100) and patients with FVIII ≤ 50% (*n* = 16)

	BUC	Healthy	*P* value	FVIII ≤ 50%	*P* value
Female, *n* (%)	332 (87)	80 (80)	0.081	7 (44)	<0.001
Blood group O, *n* (%)	178 (46.6)	37 (37)	0.082	9 (56.3)	0.454
Age	42 [29-54]	40.5 [29-50]	0.337	34 [23-62]	0.397
BMI[a]	23.3 [21.0-26.5]	22.2[20.5-25.2]	0.039	22.6 [22.1-26.2]	0.994
Bleeding score	5 [3-7]	0 [0-0]	<0.001	5 [4-6]	0.838
aPTT, s	35.5 [33.3-37.7]	35.1 [33.3-36.6]	0.186	46.0 [43.9-51.5]	<0.001
Prothrombin time, %	95 [88-103]	99 [90-109]	0.005	93 [88-98]	0.492
Fibrinogen, mg/dL	317 [277-365]	291 [244-340]	<0.001	305 [235-347]	0.242
VWF:Ag, %	100 [82-126]	104 [89-134]	0.122	67 [46-94]	<0.001
VWF:RCo, %[a]	87 [69-127]	91 [71-127]	0.673	62 [37-113]	0.002
FVIII, %	128 [103-163]	128 [105-157]	0.815	26 [21-38]	<0.001
FIX, %[d]	107 [91-123]	101 [89-117]	0.077	103 [88-126]	0.595
FXIII, %	127 [110-144]	131 [115-151]	0.031	130 [98-139]	0.680
Hemoglobin, g/dL	13.5 [12.8-14.2]	13.7 [13-14.4]	0.130	13.9 [13.1-14.5]	0.506
Platelet count, $\times 10^9$/L	246 [214-284]	257 [220-296]	0.103	242 [197-341]	0.986
Leukocytes, $\times 10^9$/L	5.8 [5.0-7.1]	5.7 [5-6.7]	0.311	6.1 [4.9-7.8]	0.641
PAI-1, U/mL[b]	1.25 [0.49-3.38]	1.10 [0.49-3.50]	0.593	1.50 [0.49-2.75]	0.831
alpha2-antiplasmin, %[c]	104 [94-112]	99.0 [92.0-107.3]	0.014	99.5 [91.8-106.8]	0.207

Note: Unless otherwise stated, data are shown in median and interquartile range [25th-75th percentile]. aPTT, activated partial thromboplastin time; BMI, body mass index; BUC, bleeding of unknown cause; FIX, factor IX activity; FVIII, factor VIII activity; FXIII, factor XIII activity; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; PAI-1, plasminogen activator inhibitor-1.
[a]Available of 379 BUC patients and 99 healthy controls.
[b]Available of 344 BUC patients and 98 healthy controls.
[c]Available of 375 BUC patients and 99 healthy controls.
[d]Available of 376 BUC patients. (for FIX).

TABLE 2 Thrombin generation parameters in patients with BUC (*n* = 382) compared to healthy controls (*n* = 100) and patients with FVIII ≤ 50% (*n* = 16)

	BUC	Healthy	*P* value[a]	FVIII ≤ 50%	*P* value[b]
Thrombin generation					
Lag time, min	10.6 [9.1-12.6]	9.1 [8.1-11.1]	<0.001	13.4 [11.0-16.4]	0.031
Velocity index, nmol/L/min	33.1 [17.7-55.8]	65.8 [32.3-115.4]	<0.001	10.0 [4.9-18.6]	<0.001
Peak thrombin, nmol/L	238 [161.4-325.7]	362.5 [251.2-475.4]	<0.001	116.5 [81.1-183.9]	0.002
TTP, min	18.1 [15.1-21.6]	15.1 [12.1-18.6]	<0.001	24.9 [23.4-31.7]	<0.001
AUC, nmol/L × min	3274.5 [2849.4-3732.0]	3784.9 [3302.9-4067.1]	<0.001	2926.5 [2055.1-3270.7]	0.364

Note: Data are shown in median and interquartile range [25th-75th percentile]. AUC, area under the curve; BUC, bleeding of unknown cause; FVIII, factor VIII activity; min, minutes; TTP, time to peak.
[a]Comparison of BUC patients and healthy controls, adjusted for fibrinogen, factor XIII, and BMI by multiple linear regression analysis.
[b]Comparison of BUC and FVIII ≤ 50% patients, adjusted for sex by multiple linear regression analysis.

and BUC patients showed a lower velocity index, decreased peak thrombin, longer TTP, and decreased AUC compared to healthy controls. Comparison of BUC patients to patients with FVIII ≤ 50% showed significant differences in thrombin-generation parameters, with a shorter lag time, higher velocity index, higher peak thrombin, and shorter TTP. The AUC was equally impaired in patients with BUC and FVIII ≤ 50%.

3.3 | Clot formation and lysis in patients with BUC compared to healthy controls and patients with FVIII deficiency

Patients with BUC showed significantly altered clot formation, including a lower clot formation rate (Vmax), increased ΔAbs, prolonged TTP, and shorter CLT compared to healthy controls (Table 3,

TABLE 3 Clot formation and lysis parameters in patients with BUC (*n* = 382) compared to healthy controls (*n* = 100) and patients with FVIII ≤ 50% (*n* = 16)

	BUC	Healthy	*P* value[a]	FVIII ≤ 50%	*P* value[b]
Plasma clot properties					
Lag time, min	10.4 [7.3-13.8]	9.3 [7.7-12.2]	0.730	13.0 [10.4-21.5]	0.035
Vmax, OD/min	0.13 [0.10-0.17]	0.16 [0.12-0.20]	<0.001	0.10 [0.07-0.12]	0.003
ΔAbs, OD_{405nm}	0.54 [0.44-0.64]	0.49 [0.42-0.60]	<0.001	0.43 [0.34-0.59]	0.032
TTP, min	19.5 [14.4-23.5]	16.0 [13.7-19.7]	0.029	22.2 [17.2-30.3]	0.018
CLT, min	16.1 [13.5-19.7]	18.2 [14.9-22.4]	<0.001	14.5 [12.7-16.8]	0.384

Note: Data are shown in median and interquartile range [25th-75th percentile]. BUC, bleeding of unknown cause; CLT, clot lysis time; FVIII, factor VIII; min, minutes; TTP, time to peak.

[a]Comparison of BUC patients and healthy controls, adjusted for fibrinogen, factor XIII und BMI by multiple linear regression analysis.

[b]Comparison of BUC and FVIII ≤50% patients, adjusted for sex by multiple linear regression analysis.

Figure S2). There was no difference in the lag time between patients with BUC and healthy controls. In patients with FVIII deficiency, clot formation and lysis were significantly impaired in comparison to healthy controls (Table S3). Clot formation was even more impaired than in BUC patients, as indicated by a longer lag time, lower clot formation rate (Vmax), lower ΔAbs, and longer TTP. There was no

TABLE 4 Correlation of thrombin-generation parameters with clinical and laboratory parameters in patients with BUC (*n* = 382) and healthy controls (*n* = 100)

	Lag time, min	Velocity index, nmol/L/min	Peak thrombin, nmol/L	TTP, min	AUC, nmol/L × min
Patients BUC					
Age, years	0.128	0.094	0.064	0.003	−0.066
BMI, kg/m^2	0.015	0.113	0.154	−0.021	0.250
Bleeding score	0.077	−0.037	−0.036	0.060	−0.017
aPTT, s	0.311	−0.336	−0.325	0.349	−0.273
Prothrombin time, %	0.007	0.010	0.030	0.018	0.079
Fibrinogen, mg/dL	−0.045	0.279	0.320	−0.135	0.343
VWF:Ag	−0.086	0.246	0.228	−0.187	0.161
VWF:RiCo	−0.093	0.210	0.191	−0.174	0.126
FVIII activity, %	−0.143	0.319	0.299	−0.260	0.192
FIX activity, %	−0.119	0.299	0.323	−0.197	0.301
FXIII activity, %	0.008	0.122	0.138	−0.050	0.006
Controls					
Age, years	0.023	0.091	0.074	−0.037	0.022
BMI, kg/m^2	0.079	0.042	0.070	0.042	0.084
aPTT, s	0.284	−0.320	−0.314	0.312	−0.313
Prothrombin time, %	−0.246	0.238	0.259	−0.226	0.223
Fibrinogen, mg/dL	0.019	0.118	0.170	−0.004	0.315
VWF:Ag, %	−0.016	0.122	0.130	−0.058	0.152
VWF:RiCo, %	−0.123	0.233	0.243	−0.169	0.244
FVIII activity, %	−0.158	0.202	0.220	−0.180	0.252
FIX activity, %	−0.204	0.336	0.367	−0.252	0.365
FXIII activity, %	−0.157	0.198	0.248	−0.164	0.243

Note: Weak (*r* = 0.2-0.4), moderate (*r* = 0.4-0.6), and strong (*r* = 0.6-0.8) and very strong (*r* = 0.8-1.0) correlation.

Abbreviations: aPTT, activated partial thromboplastin time; AUC, area under the curve; BMI, body mass index; BUC, bleeding of unknown cause; FIX, factor IX activity, FVIII, factor VIII activity; FXIII, factor XIII activity; TTP, time to peak; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity.

difference in CLT between patients with FVIII ≤ 50% and BUC patients (Table 3).

3.4 | Correlation of thrombin-generation parameters with clinical and laboratory parameters of patients with BUC and healthy controls

To identify a possible influence of clinical and laboratory characteristics on thrombin generation and clot formation and lysis, we calculated correlations in patients with BUC and healthy controls (Table 4 and Table 5). Thrombin generation parameters showed only weak correlations with clinical and laboratory characteristics. Both BUC patients and controls showed a weak, but consistent correlation between the aPTT and all thrombin-generation parameters, whereas the prothrombin time correlated with these parameters only in the control group. In both BUC patients and controls, FVIII and FIX activity correlated weakly with peak thrombin and velocity index, but not with the lag time. Fibrinogen and VWF:Ag correlated weakly with peak thrombin, velocity index, and AUC in the BUC patients, but not controls.

3.5 | Correlation of clot parameters with clinical and laboratory parameters of patients with BUC and healthy controls

We found weak to strong correlations between clinical and laboratory parameters and parameters of clot formation and lysis (Table 5). Fibrinogen showed the strongest correlation with plasma clot parameters, especially with Vmax and ΔAbs. Factor VIII, FIX, and FXIII activity also correlated weakly to moderately with clot turbidity (ΔAbs), clot formation rate (Vmax), and CLT in patients with BUC and controls. Neither aPTT nor PT showed any consistent correlations with plasma clot parameters in either BUC patients or controls. There was also no correlation between bleeding score and clot formation or lysis parameters.

3.6 | Cut off values of thrombin generation, clot formation, and clot lysis parameters

We also analyzed thrombin generation, clot formation, and clot lysis parameters using cutoffs defined by the 25th/75th and

TABLE 5 Correlation of plasma clot parameters with clinical and laboratory parameters in patients with BUC (n = 382) and healthy controls (n = 100)

	Lag time, min	Vmax, OD/min	ΔAbs, OD_{405nm}	TTP, min	CLT, min
Patients BUC					
Age, years	0.072	0.136	0.237	0.066	0.215
BMI, kg/m^2	0.170	0.098	0.268	0.165	0.207
Bleeding score	0.018	0.085	0.111	0.024	0.029
aPTT, s	0.130	−0.178	−0.100	0.192	−0.019
Prothrombin time, %	−0.083	0.195	0.215	−0.101	0.219
Fibrinogen, mg/dL	−0.024	0.536	0.870	0.030	0.380
VWF:Ag, %	−0.026	0.217	0.312	−0.044	0.147
VWF:RiCo, %	−0.020	0.168	0.263	−0.037	0.068
FVIII activity, %	−0.098	0.295	0.373	−0.105	0.212
FIX activity, %	−0.021	0.377	0.507	−0.052	0.257
FXIII activity, %	0.060	0.221	0.403	0.069	0.327
Controls					
Age, years	0.085	0.158	0.399	0.167	0.165
BMI, kg/m^2	0.103	0.225	0.384	0.120	0.321
aPTT, s	0.303	−0.175	−0.160	0.310	−0.299
Prothrombin time, %	−0.132	−0.006	0.196	−0.070	0.300
Fibrinogen, mg/dL	0.067	0.461	0.877	0.180	0.320
VWF:Ag, %	−0.011	0.303	0.377	0.016	0.128
VWF:RiCo, %	−0.081	0.288	0.324	−0.035	0.207
FVIII activity, %	−0.124	0.420	0.437	−0.092	0.277
FIX activity, %	0.018	0.334	0.474	0.003	0.322
FXIII activity, %	−0.179	0.173	0.337	−0.079	0.462

Note: Weak (r = 0.2-0.4), moderate (r = 0.4-0.6), and strong (r = 0.6-0.8) and very strong (r = 0.8-1.0) correlations.
Abbreviations: aPTT, activated partial thromboplastin time; BMI, body mass index; BUC, bleeding of unknown cause; CLT, clot lysis time; FIX, factor IX activity; FVIII, factor VIII activity; FXIII, factor XIII activity; TTP, time to peak; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity.

5th/95th percentiles of healthy controls (Table S4). For all analyzed parameters, values above or below the 25th and 75th percentiles occurred more often in patients with BUC than in healthy controls. Values above or below the 5th and 95th percentiles occurred more often in patients with BUC than in healthy controls in velocity index, peak thrombin, and AUC of thrombin generation and all parameters of plasma clot properties except ΔAbs.

3.7 | Thrombin generation and clot formation and lysis in BUC patients according to their bleeding severity

There were no correlations between parameters of thrombin generation or plasma clot formation and bleeding score (Table 4 and Table5). In a separate analysis we investigated a potential difference between patients with a "nonpathological" bleeding severity (BS below the cutoff) and patients with a bleeding score above the gender-specific cutoff (≥3 for males und ≥5 for females)[20] as well as healthy controls (Table 6). A total of 139 patients (36.4%) had a BS below and 243 patients (63.6 %) above the gender-specific cutoff. Comparison of BUC patients with an abnormal BS to BUC patients with a BS below the cutoff did not show significant differences in any of the analyzed parameters (Table 6). Moreover, all parameters of thrombin generation were still impaired in BUC patients with a bleeding score below the predefined threshold in comparison to healthy controls. Also compared to healthy controls, patients with a BS below the cutoff had a reduced clot formation rate, increased ΔAbs, and shortened clot lysis time (Table 6).

4 | DISCUSSION

In this study we observed that thrombin generation and clot formation are impaired and clot lysis time is shortened in plasmas from BUC patients compared to healthy controls. These results indicate that patients with BUC have a reduced hemostatic potential, which most likely contributes to their increased bleeding tendency. Use of these global assays might have interesting implications for both diagnosis of patients with suspected hemostatic defects and defining of the underlying mechanism.

Patients with BUC suffer from a clinically relevant bleeding tendency while showing normal test results in all routine coagulation tests.[1–3] According to our data, parameters of thrombin generation and clot formation and lysis identify patients with BUC. Thus, our results suggest the use of these assays in situations where traditional clotting tests such as aPTT and PT fail to differentiate. So far, lack of abnormalities in the laboratory assessment might prevent physicians from initiating appropriate hemostatic treatment in patients with BUC. Evidence of reduced hemostatic capacity can alert physicians about hemostatic impairment, potential risk of bleeding, and consideration of hemostatic support during hemostatic challenges such as surgery, pregnancy, and childbirth. Interestingly, bleeding severity, assessed by a standardized bleeding score, was similar in patients with BUC and FVIII deficiency and did not correlate with parameters of thrombin generation or clot formation and lysis. This observation suggests that the bleeding score does not appropriately capture the degree of hemostatic system impairment in patients

TABLE 6 Thrombin-generation parameters and clot formation and lysis parameters in BUC patients with a bleeding score below the gender-specific cutoff (≥3 for males and ≥5 for females; *n* = 139) compared to BUC patients with a bleeding score above the cutoff (*n* = 243) and healthy controls

	BUC (BS <cutoff)[c]	BUC (BS ≥cutoff)[c]	*P* value[b]	Healthy	*P* value[c]
Thrombin generation					
Lag time, min	10.1 [9.1-12.1]	11.1 [9.6-13.1]	0.316	9.1 [8.1-11.1]	0.037
Velocity index, nmol/L/min	37.4 [19.8-66.3]	31.5 [16.4-51.6]	0.931	65.8 [32.3-115.4]	<0.001
Peak thrombin, nmol/L	243.5 [175.7-352.3]	237.0 [154.8-309.5]	0.569	362.5 [251.2-475.4]	<0.001
TTP, min	17.1 [14.6-20.6]	18.6 [15.6-22.1]	0.170	15.1 [12.1-18.6]	0.001
AUC, nmol/L × min	3377 [2932.1-3836.3]	3224.5 [2795.7-3659.5]	0.067	3784.9 [3302.9-4067.1]	0.001
Plasma clot properties					
Lag time, min	10.1 [7.2-13.4]	10.6 [7.4-14.5]	0.913	9.3 [7.7-12.2]	0.891
Vmax, OD/min	0.12 [0.10-0.17]	0.13 [0.10-0.17]	0.301	0.16 [0.12-0.20]	<0.001
ΔAbs, OD_{405nm}	0.54 [0.43-0.64]	0.54 [0.44-0.64]	0.401	0.49 [0.42-0.60]	0.003
TTP, min	19.5 [14.1-22.3]	19.4 [14.6-24.0]	0.785	16.0 [13.7-19.7]	0.101
CLT, min	16.3 [13.5-19.9]	16.1 [13.5-19.6]	0.905	18.2 [14.9-22.4]	0.001

Note: Data are shown in median and interquartile range [25th-75th percentile]. ΔAbs, maximum absorbance at plateau; AUC, area under the curve; BS, bleeding score; BUC, bleeding of unknown cause; CLT, clot lysis time; min, minutes; nmol/L, nanomolar; OD, optical density; TTP, time to peak.

[a]Gender-specific cutoff defined as ≥3 for males and ≥5 for females.[20]

[b]Comparison of BUC patients below cutoff with BUC patients above cutoff, adjusted for age, sex, blood group, hemoglobin, factor VIII, and factor XIII by multiple linear regression analysis.

[c]Comparison of BUC patients below cutoff with healthy controls, adjusted for sex, hemoglobin, fibrinogen, and factor XIII by multiple linear regression analysis.

with BUC. This observation is in line with a recent meta-analysis indicating that bleeding assessment tools lack sufficient sensitivity and specificity to discriminate patients with and without mild bleeding disorders.[26] Whereas most parameters of thrombin generation and plasma clot properties correlated strongly in controls, correlations were mostly weak in BUC patients (Table S5). Therefore, the alterations in plasma clot formation in our group of BUC patients are only insufficiently explained by the decreased generation of thrombin.

The mechanisms underlying the delayed and decreased thrombin generation and plasma clot formation in our patients with BUC remain unclear. Most of the alterations that we found in BUC patients were similar, though less pronounced than those seen in FVIII-deficient patients, suggesting some of the hemostatic defect may be common to both of these groups of patients. One possible mechanism might be increased inhibitor antigen or activity,[27] which would not be detected by global coagulation tests. For example, Dargaud et al described a thrombomodulin mutation leading to elevated levels of soluble thrombomodulin, enhanced protein C activation, and impaired thrombin generation (decreased AUC) in a patient with a bleeding tendency and otherwise normal coagulation screening tests.[28] A gain-of-function mutation in Factor V that leads to increased circulating TFPI, decreased thrombin formation, and bleeding was also recently described.[29–31] In those patients, routine global tests of aPTT and prothrombin time were also affected. Altered expression of fibrinogen isoforms that have anticoagulant activity (γ')[32,33] might also modify thrombin generation and clot formation and lysis in a manner that is not detected by conventional coagulation assays. Future studies, including analysis of soluble thrombomodulin, free TFPI, and fibrinogen γ' levels, are warranted to identify the underlying mechanisms contributing to bleeding in these patients.

In a previous study of Ay et.al., we were not able to detect a difference in thrombin generation in BUC patients compared to healthy controls, which is in contrast to the current study.[10] This discrepancy might be explained by variation in patient selection and by the much smaller sample size in the previous cohort. In line with the significantly deteriorated clot formation in this study, we have also shown a slower clot formation rate (Vmax) in the presence of rtPA in a previous study on a smaller group of patients with BUC.[18] Ryan et al reported that at constant fibrinogen concentrations, low concentrations of thrombin lead to a slower formation of clots containing thicker fibrin fibers. Thus, the reduced thrombin generation might underlie the slower formation and increased turbidity of the clots in our BUC patient group. We found a shorter CLT, indicating increased susceptibility of the clots of BUC patients to lysis, which has previously also been observed in a group of women with heavy menstrual bleeding without known hemostatic abnormalities.[34] Moreover, in a previous investigation of fibrinolysis parameters in plasmas from patients with BUC we showed that increased fibrinolysis might underlie the bleeding tendency in at least some of the patients.[35] The findings of increased clot fibrinolytic susceptibility in patients with BUC are also supported by the clinical efficacy of antifibrinolytic agents such as tranexamic acid for treating mild to moderate bleeding symptoms.[36–38]

Our study has several limitations. First, the bleeding phenotype of the investigated patients was mild and according to the bleeding score cutoffs (abnormal bleeding score: adult men ≥3, adult women ≥5), 36.4% of our patients with BUC would not be considered to have a bleeding disorder.[20] However, there was no difference in thrombin generation or clot formation and lysis between patients below and above this cutoff, whereas patients with a defined nonpathological bleeding score still had significantly impaired results compared to healthy controls (Table 6). This is further underlined as there was no difference in the bleeding severity between our group of patients with BUC and patients with FVIII activity ≤50%. Second, we were able to include only a small group of patients with FVIII ≤50% for comparison. This is due to the strict and specific inclusion criteria of the VIBB, which recruits only patients with a bleeding tendency without a previous diagnosis of an established bleeding disorder. Third, the tests performed are nonphysiological *ex vivo* tests and therefore do not completely reflect the clotting cascade *in vivo*. However, prior studies have associated parameters from these assays with clinically relevant outcomes.[9,11–15] Finally, we measured dynamic changes in plasma clot formation and lysis, but we did not explicitly investigate clot structure by scanning electron microscopy or clot permeability because of limited sample volume. Thus, refined plasma clot properties in the setting of mild bleeding disorders have yet to be thoroughly characterized.

In conclusion, our data show a reduced hemostatic potential and increased susceptibility to clot lysis in patients with BUC. Thrombin-generation parameters, the clot formation rate, and clot lysis time distinguished patients with a mild to moderate bleeding tendency without diagnosis of an established bleeding disorder from healthy controls. The comparison to patients with a known impairment of thrombin generation and plasma clot formation (patients with FVIII ≤50%) validates our assessments and supports the conclusion of a dysfunctional process of coagulation in our patients with BUC. These assays might alert physicians to the hemostatic impairment in patients with otherwise normal coagulation test results and raise awareness of an increased bleeding risk, especially in situations of hemostatic challenges. Our data warrant further investigations on the mechanisms underlying the reduced hemostatic potential and accelerated clot lysis in our patients with BUC.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

I. Pabinger, S. Hofer, J. Gebhart, and C. Ay designed the study; I. Pabinger, S. Hofer, J. Gebhart, C. Ay, and J. Rejtö recruited patients; H.Haslacher processed and stored the samples; S. Hofer and S. Koder designed and performed the experiments; S. Hofer performed statistical analyses; I. Pabinger, S. Hofer, J. Gebhart analyzed the data; I. Pabinger, S. Hofer, J. Gebhart, C. Ay, and AS. Wolberg interpreted the data; S. Hofer and J. Gebhart wrote the manuscript, which was reviewed, edited, and finally approved by all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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