

EXPLORING BLEEDING DISORDERS OF UNKNOWN CAUSE

Novel Perspectives from Global and Flow-Based Hemostasis Assays

AMAURY MONARD

Stellingen behorend bij het proefschrift

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1. Normale standaard hemostase testen sluiten een klinisch relevante stollingsstoornis niet uit. *(dit proefschrift)*
2. De diagnose Bleeding Disorder of Unknown Cause vereist zowel een klinisch relevante bloedingsneiging als een gestandaardiseerde diagnostische evaluatie. *(dit proefschrift)*
3. Gespecialiseerde plaatjesfunctietesten zijn complementair aan, maar geen vervanging voor, light transmission aggregometrie. *(dit proefschrift)*
4. De beoordeling van de primaire hemostase onder flowcondities levert aanvullende informatie ten opzichte van statische laboratoriumtesten. *(dit proefschrift)*
5. Diagnostische duidelijkheid heeft waarde, ook zonder therapeutische consequenties. *(impact paragraaf)*
6. Innovatieve hemostase testen vergroten vooral ons begrip van Bleeding Disorder of Unknown Cause; hun plaats in de klinische diagnostiek moet nog worden vastgesteld. *(impact paragraaf)*
7. Het grootste deel van de nog onbekende stollingsstoornissen zal waarschijnlijk niet met één nieuwe laboratoriumtest worden ontdekt.
8. Onze kennis van de hemostase is fysiologisch, maar onze diagnostiek is dat nog onvoldoende.
9. How wonderful that we have met with a paradox. Now we have some hope of making progress. *(Niels Bohr)*
10. If you never bleed, you're never gonna grow. *(Taylor Swift)*

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“Somewhere, something incredible is waiting to be known.”

- Carl Sagan



CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

HEMOSTASIS AND BLEEDING

The process of hemostasis consists of primary and secondary hemostasis and fibrinolysis. Platelet adhesion, platelet activation and platelet aggregation are different mechanisms of primary hemostasis, leading to the initial platelet plug formation. When endothelial damage or activation occurs, von Willebrand Factor (vWF) binds to the GPIb receptor on platelets and this results in platelet activation. The activated platelets will change their shape and release their dense storage granules, containing ATP, ADP, serotonin and Ca^{2+}, and α-granules containing vWF, factor (F) V and fibrinogen. Release of these granules causes activation of other platelets and activation of the GpIIB/IIIa receptor. The activated GpIIb/IIIa receptor on the platelet then binds to fibrinogen [1] leading to the formation of the primary platelet plug (figure 1). When platelets are activated, they undergo a plasma membrane transformation, also called the flip-flop mechanism. The inner leaflet of the plasma membrane, containing phosphatidylserine (PS), is transferred to the outer leaflet of the membrane, creating a procoagulant surface on which the coagulation cascade can take place [2].

Secondary hemostasis results in the formation of thrombin (figure 2). Initially, the coagulation cascade was considered to consist of two separate pathways, also referred to as the intrinsic and extrinsic coagulation pathway [1, 3]. According to this model, the intrinsic pathway is activated by contact activation. In vitro, this is caused by negatively charged surfaces of the processing equipment. Activation of the intrinsic pathway causes FXII to be transformed to FXIIa (activated FXII). By activation of FXII, a cascade is induced, activating FXI, FIX and FX. To initiate the extrinsic pathway, tissue factor (TF) is needed to activate FVII to FVIIa. The complex formed by TF, FVIIa and Ca^{2+} is then able to activate FX (FXa). FXa is able to activate more FVII (FVIIa), accelerating the start of coagulation. FXa also initiates the common pathway in which it forms a complex with FVa and Ca^{2+}. This complex is able to convert prothrombin into thrombin. Thrombin at its turn is able to form fibrin monomers from fibrinogen [1,5]. Fibrin fibers then form cross-linked fibrin networks, which results in a more stable clot than the initial plug formed by primary hemostasis. More recently, it has become clear that in vivo, the intrinsic and extrinsic pathway are not two separate pathways, but an integrated pathway with an initiation phase and amplification loop, leading to normal hemostasis in vivo. The most important trigger for the pathway is exposure of TF due to endothelial cell damage [3].

Eventually, the formed clot has to be broken down and that is where the fibrinolytic system comes into play. Plasmin has a key role in the fibrinolytic process and is strictly controlled by different activators and inhibitors. The first step into the fibrinolytic pathway is the conversion of plasminogen into plasmin, which is stimulated by tissue plasminogen activator (tPA) and urokinase (uPA) and inhibited by plasminogen activator inhibitor-1

and 2 (PAI-1 and 2), α2-antiplasmin (A2AP) and thrombin activatable fibrinolysis inhibitor (TAFI). Plasmin is then able to degrade fibrin threads into fibrin degradation products (figure 1) [4].

Hemostasis is a well controlled process in which numerous feedback mechanisms are designed to prevent a patient from thrombotic or bleeding events [3]. In case of insufficient potency of the hemostatic system to occlude a disrupted blood vessel, hemorrhagic episodes may occur. Hemostatic failure can be caused by a congenital or an acquired bleeding disorder, or other causes apart from the hemostasis system such as liver and kidney disease, hypothyroidism and medication use [5]. However, the large majority of patients with bleeding symptoms who are referred to a hemostasis specialist remains undiagnosed [6]. A variety of laboratory hemostasis assays is available to investigate for hemostatic abnormalities in bleeding patients, as different bleeding disorders require different treatment and management regimens.

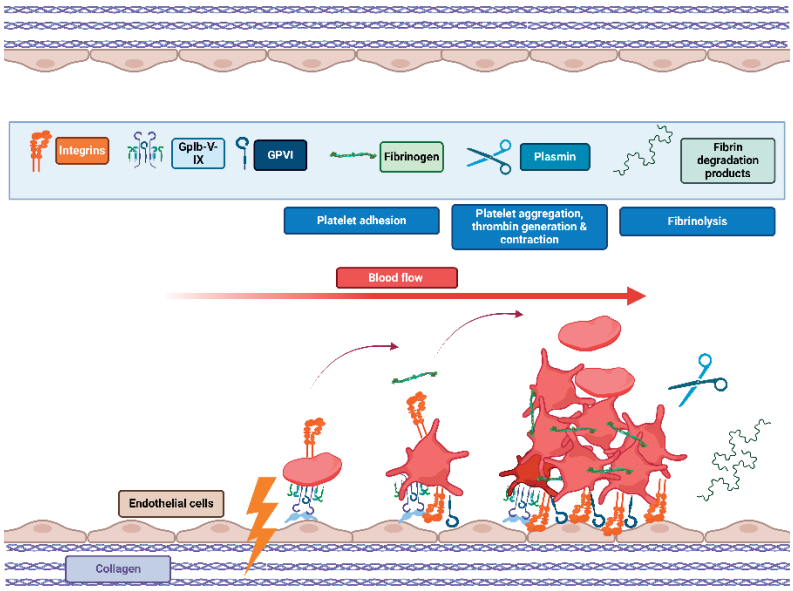

Figure 1: schematic overview of the primary hemostasis and fibrinolytic process. Figure created with biorender.

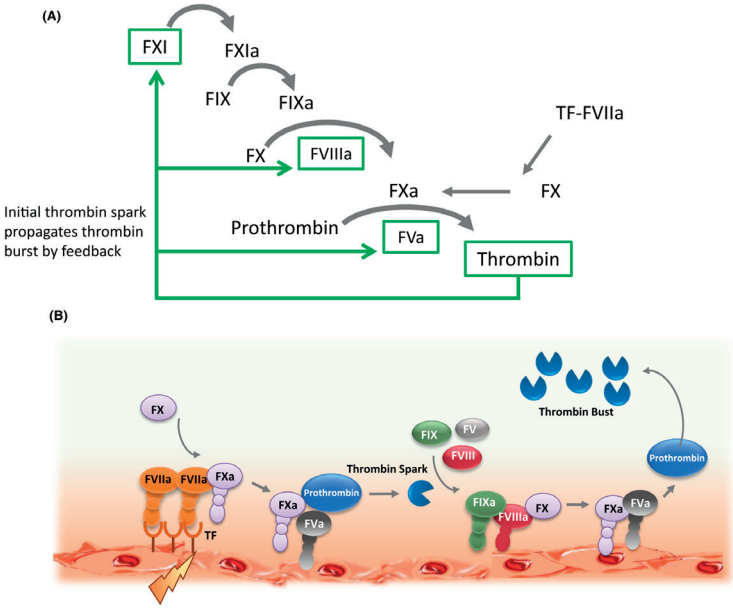

Figure 2: schematic overview of the secondary hemostasis process, reprinted with permission [3].

DIAGNOSTIC WORK-UP OF A BLEEDING TENDENCY

Assessment of the bleeding history

When a patient with an increased bleeding tendency is referred to a medical specialist for diagnostic work-up of their bleeding symptoms, a distinction must be made between trivial bleeding symptoms and symptoms that may indicate an underlying bleeding disorder. This distinction is complex for several reasons, including the subjective nature of bleeding reports and the fact that bleeding symptoms are also regularly reported in the healthy population [7-9]. Various questionnaires and scoring systems have been developed to quantify bleeding symptoms more objectively, known as bleeding assessment tools (BAT). A commonly used BAT, developed by the International Society on Thrombosis and Haemostasis (ISTH), is the ISTH-BAT, in which an abnormal bleeding score is defined as a score of >4 for men, >6 for women, and >3 for children [10, 11].

However, BAT's have their limitations [12]. The BAT score may be incorrectly low in, for example, children and young adults who have usually not yet been exposed to situations where adequate hemostasis is required (surgeries, dental procedures, traumas and childbirth). Therefore, adjusted reference ranges for the ISTH-BAT based on age and sex were published more recently, resulting in a lower upper reference value in younger

persons [13, 14]. An incorrect normal BAT score can also occur in families where an increased bleeding tendency has been present for generations, and severe bleeding symptoms, such as during menstruation, are considered normal [7, 13, 15]. Additionally, the family history is not included in the BAT score.

Another significant shortcoming is that mainly the severity of the bleeding is evaluated, not the frequency of bleeding. In the cases mentioned above, the clinical judgment of a physician with expertise is important. The expert determines in daily practice whether there is, despite a low BAT score, an abnormal bleeding tendency that is clinically relevant or interferes with the patient's daily life ("nuisance bleeding"). Lastly, it may happen that there is an increased BAT score based on a single very severe bleeding occurrence after surgery or childbirth, where a surgical or other clinical explanation for the bleeding is more plausible [6]. Therefore, to establish an abnormal bleeding tendency, it is important not only to consider the BAT score but also to take the clinical judgment of the physician into account. An expert physician can weigh all factors that influence the likelihood of a bleeding disorder, including family history, other causes of bleeding, medication use, age, and medical history [16].

Laboratory diagnostics

Laboratory testing is essential in diagnosing a bleeding disorder. Various diagnostic algorithms have been described in literature for conducting laboratory tests in patients suspected for a bleeding disorder. Generally, a stepwise approach is used, beginning with screening hemostasis tests for primary and secondary hemostasis, such as the platelet function analyzer (PFA) for von Willebrand disease (VWD) and platelet function defects (PFD), and the activated partial thromboplastin time (aPTT) and prothrombin time (PT) for coagulation factor deficiencies [5, 16, 17]. These screening tests may fail to detect mild abnormalities. Additionally, fibrinogen levels can be measured. A complete blood count, including platelet count, is also part of the screening process. For detecting VWD, von Willebrand factor activity and antigen levels are measured (table 1).

Additional laboratory tests can be performed in the next step based on the results of the screening tests, the clinical judgment of the physician, ISTH-BAT score and the likelihood of an underlying bleeding disorder. To diagnose coagulation factor deficiencies, individual factors (FII, FV, FVII, FVIII, FIX, FX, FXI, and FXIII) can be measured, as recommended in guidelines for rare bleeding disorders [18]. For VWD, if vWF activity and antigen levels are abnormal, further tests such as multimer analysis and a collagen-binding assay (specific for VWD type 2M) can be conducted [19]. For diagnosing platelet function disorders, light transmission aggregometry (LTA) is the gold standard. Further investigation into storage pool diseases can be done using ATP release assays or electron microscopy [20]. Lastly, flow cytometry can also be utilized to test for specific platelet receptor defects (table 1).

Table 1: Overview of hemostasis tests.

Screening hemostasis tests	Complete blood count, MPV, PT, aPTT, TT, fibrinogen, PFA-200
Von Willebrand tests (screening)	vWF activity and vWF antigen
Von Willebrand tests (additional)	VWF multimer analysis, collagen binding assay
Coagulation factors	FII, FV, FVII, FVIII, FIX, FX, FXI, FXIII
Platelet function tests	LTA, platelet secretion assay, electron microscopy, flow cytometry

MPV, mean platelet volume; PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time; PFA-200, platelet function analyzer 200; vWF, von Willebrand Factor; LTA, light transmission aggregometry.

There are also conditions associated with an increased bleeding tendency without abnormalities in the coagulation cascade, such as connective tissue disorders, liver and kidney diseases, and hypothyroidism [5]. To rule out other causes, additional laboratory tests can be performed, including C-reactive protein measurement, kidney and liver function tests, and thyroid-stimulating hormone (TSH) and free T4 (FT4) measurements. A hypermobility screening can also be conducted, and non-accidental trauma should be considered.

INNOVATIVE ADVANCED LABORATORY TESTING

It is important to note that the laboratory hemostasis assays that were described in table 1 are almost all static assays using (citrated) plasma. One exception is the PFA-200, which uses wholeblood and very high shear rates to screen for primary hemostasis abnormalities. However, normal PFA-200 results do not exclude mild PFD and mild VWD [21, 22]. Another limitation of these tests is that they don't take the interaction between primary and secondary hemostasis into account, by eliminating certain elements from the whole blood. Moreover, no standardized fibrinolytic assays are available, causing a challenge in diagnosing fibrinolytic disorders. There is a strong need for (global) hemostasis assays, incorporating multiple aspects of the hemostastic process, such as the vessel wall, shear rates and the interplay between primary and secondary hemostasis, to better mimic the in vivo situation. Several innovative and advanced hemostasis assays have been developed but most of them still have to find their way to the clinical diagnostic laboratories. The innovative or advanced hemostasis assays that will be discussed in this thesis are the tissue plasminogen activator rotational thromboelastometry (tPA-ROTEM), the thrombin generation assay (TGA), advanced platelet function tests including platelet secretion assays and flow cytometry based assays, and the Maastricht flow chamber model.

tPA-ROTEM

Although most fibrinolytic proteins can be quantified in the lab, their activity levels do not reflect the overall fibrinolytic state of a patient. Global hemostasis assays, including thromboelastometry, assess changes in viscosity and elasticity during the clotting process using whole blood. These circumstances better resemble the in vivo situation and modifications to the assays can be used to measure for example fibrinolysis [23]. The tPA-ROTEM is a global fibrinolysis assay that was developed in the Maastricht University Medical Center + [24]. It is a customized assay on the ROTEM, adding tPA to whole blood and thereby inducing fibrinolysis. Different tPA-ROTEM parameters, such as the lysis time (LT) and lysis onset time (LOT), provide information on the overall fibrinolytic profile of a patient and are able to identify hyper-, normo- and hypofibrinolytic profiles. Several studies have investigated the use of the tPA-ROTEM in different patient populations. Hypofibrinolytic profiles were found in sepsis patients and in patients after cardiothoracic surgery [24]. In a group of patients with self-reported bleeding symptoms, the tPA-ROTEM showed no altered fibrinolytic profile as compared to patients without bleeding symptoms [25] and in a part of patients with chemotherapy induced thrombocytopenia, a hyperfibrinolytic profile was seen [26]. Lastly, in a group of COVID-19 patients, a severe hypofibrinolytic profile was observed [27]. Results of these studies show that the tPA-ROTEM is applicable to different patient populations and it can be of interest in the diagnostic pathway of bleeding tendencies.

Thrombin Generation Assay (TGA)

Another global hemostasis assay is the thrombin generation assay (TGA). This assay provides information on the overall hemostatic status of a patient, measuring both thrombin generation and inhibition simultaneously. Different trigger reagents can be used to identify a hyper-, normo- or hypocoagulable state [28, 29]. In bleeding patients, a decreased thrombin generation potential might be suspected. The TGA has been applied, for instance, in research in patients with hemophilia A and B, where it was able to show a correlation between the clinical bleeding phenotype and the thrombin generation capacity [30-32]. Moreover, several studies have looked into patients with bleeding disorder of unknown cause (BDUC), showing mixed results. Some studies report a prolonged lag time, a delayed time to peak, and/or reduced maximum thrombin generation in individuals with BDUC compared to healthy volunteers [33-35]. However, other studies did not confirm these findings [36, 37]. The heterogeneity of TG methods used may contribute to these different outcomes. However, these previous results show that the TGA could play a role in the analysis of a bleeding tendency.

Advanced platelet function tests

Diagnosing a mild platelet function disorder can be challenging as oposed to severe platelet function disorders, such as Glanzmann thrombostenia and Bernard Soulier

syndrome (BSS), having a more straightforward clinical presentation. As briefly mentioned earlier in this chapter, light transmisscion aggregometry (LTA) is the gold standard for platelet function testing. However, this test can miss mild platelet function defects and storage pool disease (SPD) [38, 39]. Therefore, additional platelet function tests can be performed, such as nucleotide tests and flow cytometry based tests [40]. The addition of more specialized platelet function assays to the diagnostic algorithm of bleeding tendency analysis could possibly increase the diagnostic yield for platelet function disorders byond the LTA.

Flow chamber models

As most currently available hemostasis tests use plasma and are static, microfluidic techniques using whole blood and shear rates are an interesting tool to further look into different aspects of the hemostatic process, better mimicking the in vivo situation. A variety of hemostasis on a chip models have been described in literature [41-45]. This thesis will focus on the applicability of two flow models in bleeding disorders; the Total Thrombus formation Analysis System (TTAS) and the Maastricht flow chamber. The T-TAS is a commercially availbale flow model providing different chips to investigate thrombus formation, even in patients with thrombocytopenia. The Maastricht flow chamber is an assay that was developpped at the Biochemistry department of Maastricht University. It is a model using a coverslip coated with for example collagen I and III, and (sometimes) co-coated with tissue factor (TF). Wholeblood is used and a pump using shear rates at 1000 s^{-1} causes the blood to flow through the chamber. Realtime images are made using an EVOS microscope. Different fluorescent lables can be added to the whole blood to investigate for example PS-exposure and P-selectin expression. The system is also brought to a temperature of 37C°. Flow chamber research, including the Maastricht flow chamber, has been conducted in individuals with unexplained bleeding tendencies and abnormal PFA test results in a previous study. Compared to controls, these individuals showed reduced platelet adhesion and thrombus formation, along with decreased integrin activation, phosphatidylserine exposure, and P-selectin expression, suggesting a shear-dependent platelet function disorder [46]. These models are therefore a promising tool in diagnosing patients with shear-dependent hemostastic abnormalities.

Bleeding Disorder of Unknown Cause (BDUC)

Patient characteristics, bleeding phenotype and clinical relevance

Up to 40-70% of the patients who are referred to a medical specialist for bleeding tendency analysis will eventually be diagnosed with a bleeding disorder of unknown cause (BDUC) [6]. These patients have a clinically relevant increased bleeding tendency, without any abnormalities in a set of laboratory hemostasis tests [6]. More than 60% of individuals with BDUC are female, and the diagnosis is usually made at an age of 30 to 40 years [6, 9, 47]. Symptoms such as heavy menstrual bleeding (HMB) and postpartum hemorrhage (PPH)

are common and result in a higher bleeding tendency at a younger age [6, 9]. Additionally, it is notable that 30-40% of individuals with BDUC have a positive family history [6]. This makes an underlying genetic background more likely in this patient group.

Studies show that the bleeding pattern of individuals with BDUC is characterized by mucocutaneous bleeding, such as menorrhagia, hematomas, and epistaxis, as well as bleeding during and/or after surgeries and dental procedures [9]. The bleeding pattern, the severity of the bleeding, and the BAT score in BDUC differ little, if at all, from persons who do have a clear diagnosis, such as Von Willebrand disease (VWD), platelet function disorders (PFD) and (mild) coagulation factor deficiencies [9]. Due to the lack of knowledge on the underlying pathophysiological mechanism in BDUC, there are still no evidence-based guidelines regarding risk situations, as well as for genetic counseling [16] [5, 48]. Therefore, it is not surprising that, similar to individuals with a diagnosis, those with BDUC can experience increased morbidity and a decreased quality of life, among other issues due to uncertainty and anxiety surrounding bleeding risk and management of bleeding and procedures [49]. One previous study has shown that this can also lead to a decreased quality of life in these patients [49]. Additionally, surgical or dental interventions could be postponed due to the lack of a clear diagnosis and there could be an overuse of preventative hemostatic agents, leading to higher morbidity and higher healthcare costs [5]. Therefore, it is important to recognize and diagnose BDUC as an entity. This ensures the individual is registered, and if indicated, followed up after diagnosis. This way, there is a contact point for patients when problems arise, a treatment plan can be developed when procedures or other risky situations occur, and lifestyle advice can be provided. Furthermore, awareness of the presence of a bleeding disorder develops within families [16, 50].

BDUC definition

For a long time, both internationally and within the Dutch hemophilia treatment centers (HTCs), different definitions of BDUC have been used, as well as different diagnostic and therapeutic approaches regarding this group. This explains the heterogeneity of treatment plans and the perceived urgency to strive for more uniformity [6, 51]. The commenly used definitions are heterogeneous and are not applied uniformly. BDUC is defined, among other things, as the presence of a mild to moderate bleeding tendency, and in some cases even severe, where no underlying bleeding disorder can be found after extensive investigation with currently available hemostasis tests [5, 16, 33, 34]. To be able to speak of BDUC, it is therefore important to define when there is an increased bleeding tendency and to reach consensus on which laboratory tests must be conducted as a minimum before the diagnosis of BDUC can be made. Other relevant causes must also be consciously ruled out, as BDUC is a diagnosis by exclusion. A list of BDUC definitions used in literature is shown in figure 3. In general, all these definitions include two important

BDUC definitions from the literature

Cohort	BDUC definition
Quiroga et al. 2007	No identifiable disease, even after repeated testing
Gebhart et al. 2017	Patients who are referred for evaluation of bleeding tendency with normal results in the haemostatic investigations.
Hofer et al. 2019	Patients with mild to moderate bleeding tendency in whom no underlying bleeding disorder can be identified.
Relke et al. 2019	Patients with mucocutaneous bleeding symptoms in which no underlying coagulation defect is found despite extensive laboratory testing.
Rodeghiero et al. 2019	A bleeding disorder fitting the definition of mild/moderate bleeding phenotype (but even possibly severe in some cases) that cannot be associated to any hemostatic or genetic abnormality after extensible investigation with currently available techniques. It cannot be excluded that under this provisional category future novel techniques may identify new disease entities.
Zegers et al. 2019	Patients without a final diagnosis (after diagnostic trajectory) and a high BAT score (≥3 for men and ≥5 for women)
MacDonald et al. 2019	UBD (unclassified bleeding disorder): patients with a bleeding tendency but normal standard laboratory tests.
Veen et al. 2020	Patients with a clinically relevant bleeding phenotype without a diagnosis after routine diagnostic laboratory tests.
Thomas et al. 2020	Bleeding of unknown cause has been defined as an elevated bleeding assessment tool score with normal diagnostic work-up and a significant bleeding history with no reproducible abnormality with the standard tests of hemostasis.
Mehic et al. 2021	Patients with mild-to-moderate bleeding tendency who remain without diagnosis after thorough haemostatic investigations of plasmatic coagulation and platelet function.
Baker et al. 2021	BDUC is a diagnosis by exclusion. Patients with bleeding phenotype and no abnormalities in first and second-line hemostatic tests.
Heubel-Moenen et al. 2022	Abnormal ISTH BAT score and normal standard coagulation tests
Valke et al. 2022	Tosetto bleeding assessment tool (BAT) score ≥10 and no definite diagnosis after performing primary and secondary coagulation assays.

Figure 3: BDUC definitions used in literature.

aspects; (i) the presence of a clinically relevant increased bleeding and (ii) the absence of abnormal test results in a set of laboratory hemostasis tests.

In 2024, the International Society of Thrombosis and Haemostasis published a communication letter on a standardized BDUC definition. This definition states that BDUC patients are patients referred for expert assessment of a possible bleeding tendency, with a high suspicion based on medical history (including clinical gestalt, hemostatic challenges, medication history, family history, ISTH-BAT score and physical examination) and without abnormal test results in a set of laboratory hemostasis tests (table 2) [40]. The formulation of this definition was, amongst others, based on results from an international BDUC survey [52]. Simultaneously with the international survey, we conducted a national survey in the Netherlands to investigate current practice regarding BDUC of which the results are presented in this thesis.

Table 2: Set of laboratory hemostasis tests that should be performed in BDUC patients according to the ISTH SSC [40].

Set of laboratory hemostasis assays required for BDUC diagnosis	Complete blood count, PT, aPTT, TT, Clauss fibrinogen, vWF antigen and activity, FVIII, FIX, FXI, LTA
Additional tests (if available and indicated)	Platelet secretion assays, platelet assays, fibrinolysis assays, rare clotting factor deficiencies and other highly specialized assays and targeted genomic testing

PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time; vWF, von Willebrand Factor; LTA, light transmission aggregometry.

The lack of a clear cause for the bleeding tendency in BDUC patients supports the hypothesis that this is a heterogeneous group with multiple underlying bleeding mechanisms. These may include rare monogenic disorders or polygenic bleeding tendencies [6, 47]. Given this variability, a single diagnostic test is unlikely to explain all cases of BDUC, highlighting the need for a more personalized diagnostic approach [6]. Therefore, the use of more advanced laboratory hemostasis tests, that are often still used exclusively in research settings, could contribute to solving some pieces of the BDUC puzzle. In addition to the standard hemostasis laboratory tests, various research groups have been exploring new and experimental tests to better characterize individuals with BDUC and uncover potential underlying mechanisms contributing to their bleeding tendency. The laboratory techniques that seemed promising and relevant to the BDUC population and were investigated in this thesis are tPA-ROTEM, thrombin generation, advanced platelet function tests and Maastricht Flow chamber. It is important to note that most of these tests are used in a research setting and still require harmonization and standardization.

AIM AND OUTLINE OF THIS THESIS

This thesis aims to characterize bleeding disorders of unknown cause (BDUC) and uncover the underlying pathophysiological bleeding mechanisms through advanced testing methods. **Part I** focusses on context and clinical practice regarding BDUC and the identification of BDUC knowledge gaps. In **Part II**, innovative assays were applied to explore the explanatory bleeding mechanism in this patient population.

Chapter 1 provides an introduction into hemostasis and bleeding tendency analysis, incorporating the need for laboraty hemostasis testing and the use of more advanced hemostasis assays in patients with bleeding disorders and patients with BDUC. **Chapter 2** dives deeper into the current knowledge on BDUC and its accompanying knowledge gaps in the form of an illustrated review. The problem definition of BDUC is discussed, as well as challenges regarding the diagnostic pathway, the underlying pathophysiological bleeding mechanism and the quality of life in these patients. **Chapter 3** describes the results of a survey on current practice regarding BDUC in the Netherlands. Because of the heterogeneity in BDUC definitions and clinical management of these patients, both nationally and internationally, this survey was conducted amongst physicians, nurse practitioners and clinical chemists working in Dutch hemophilia treatment centers, aiming to investigate Dutch current practice regarding BDUC and to agree on a BDUC definition.

Part II of this thesis focusses on advanced hemostasis assays. **Chapter 4** provides a scoping review on the utility of the total thrombus analysis system (T-TAS) in different clinical situations, including bleeding disorders. The T-TAS is a commercially available microfluidic model claimed to be applicable both in thrombotic and bleeding situations. The aim of this chapter was to investigate whether this assay could be of interest for patients with known bleeding disorders and BDUC patients.

In **chapter 5**, the use of the tPA-ROTEM in BDUC patients was investigated and results on their fibrinolytic profile were provided. It is hypothesized that a hyperfibrinolytic profile measured by tPA-ROTEM could explain the bleeding tendency in a part of the BDUC patients. **Chapter 6** focusses on the use of the thrombin generation assay (TGA) in BDUC patients, investigating the sensitivity of different trigger reagents in this specific patient population. The TGA reflects the balance between pro- and anticoagulant forces as it measures thrombin generation and inhibition simultaneously. In **chapter 7,** the goal was to investigate the diagnostic yield of different platelet function tests, including LTA, platelet ADP content and flow cytometry based tests in a group of patients suspected for a primary hemostasis defect. **Chapter 8** reports on the results from the Maastricht Flow chamber, a microfluidic model, that was used to investigate flow-dependent thrombus formation in BDUC patients. In **chapter 9**, a case report describes a family with hypodysfibrinogenemia

and a novel *FGG* mutation, in whom the TGA, proteomics and the Maastricht Flowchamber provided information that could not be obtained with standard hemostasis tests. This chapter depicts the broader applicability of these innovative assays byond BDUC.

Chapter 10 provides an overview and discussion on the results that were obtained in this thesis, including suggestions for future research and clinical implementation.

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PART I

SKETCHING THE PUZZLE:

Clinical and Methodological Perspectives on BDUC



CHAPTER 2

BLEEDING DISORDER OF UNKNOWN CAUSE

an illustrated review on current practice, knowledge gaps and future perspectives

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* Shared first authorship

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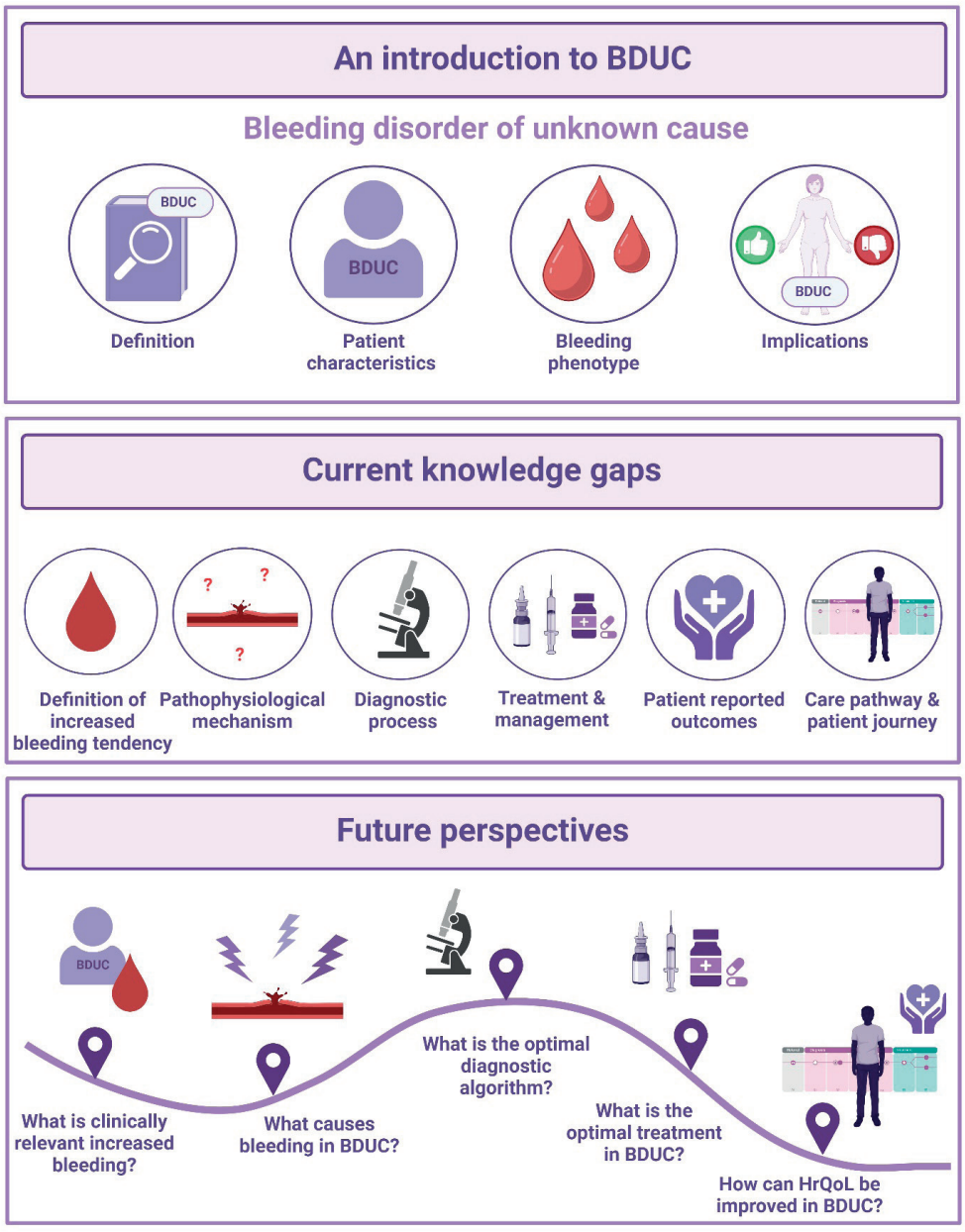
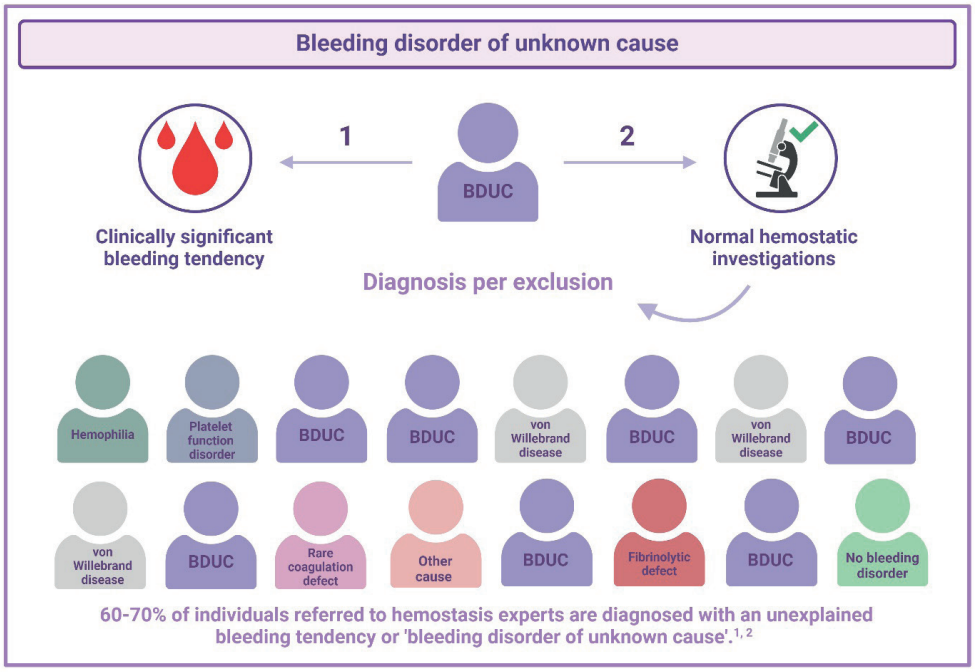
ABSTRACT

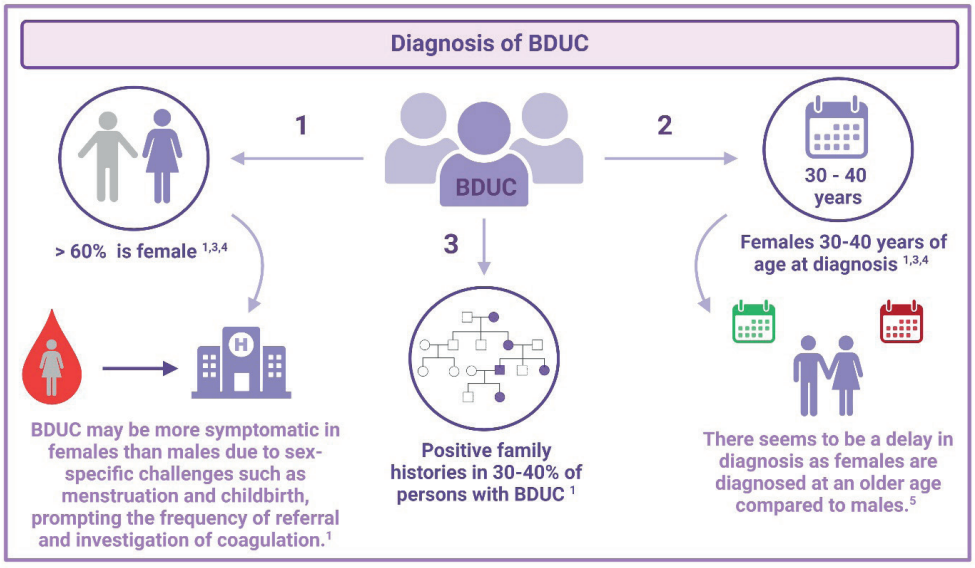
In more than half of the individuals with a clinically relevant bleeding tendency who are referred to hemostasis experts, no biological etiology can be found after extensive laboratory testing. These persons are diagnosed with an unexplained bleeding tendency or 'bleeding disorder of unknown cause' (BDUC). The mucocutaneous bleeding phenotype of individuals with BDUC is generally comparable to that of individuals with inherited bleeding disorders such as von Willebrand disease or platelet function disorders. BDUC definitions applied in literature are heterogeneous, but all comprise two main criteria: (1) there is an increased bleeding tendency based on the clinical view of the physician and/or an increased bleeding score; (2) no abnormalities are found with available hemostasis laboratory tests. This is reflected in the recent published BDUC definition by the ISTH SSC, stating that BDUC is a diagnosis of exclusion, characterized by normal hemostatic investigations despite a clinically significant bleeding tendency. Importantly, other non-hemostatic and acquired causes of bleeding should be excluded, but details on exclusion criteria and associated diagnostic testing remain undefined. Patients and healthcare providers are challenged by the uncertainty and lack of formal diagnosis particularly as there is no clear consensus regarding treatment. Research on the diagnostic value of new laboratory tests in individuals with BDUC has not yet been productive. In this illustrative review, the current practice and knowledge gaps in BDUC are addressed, previous research on BDUC is outlined and future directions with outstanding questions for future research in BDUC are highlighted.

1. Table of contents for this BDUC illustrated review



2. What is Bleeding Disorder of Unknown Cause (BDUC)?



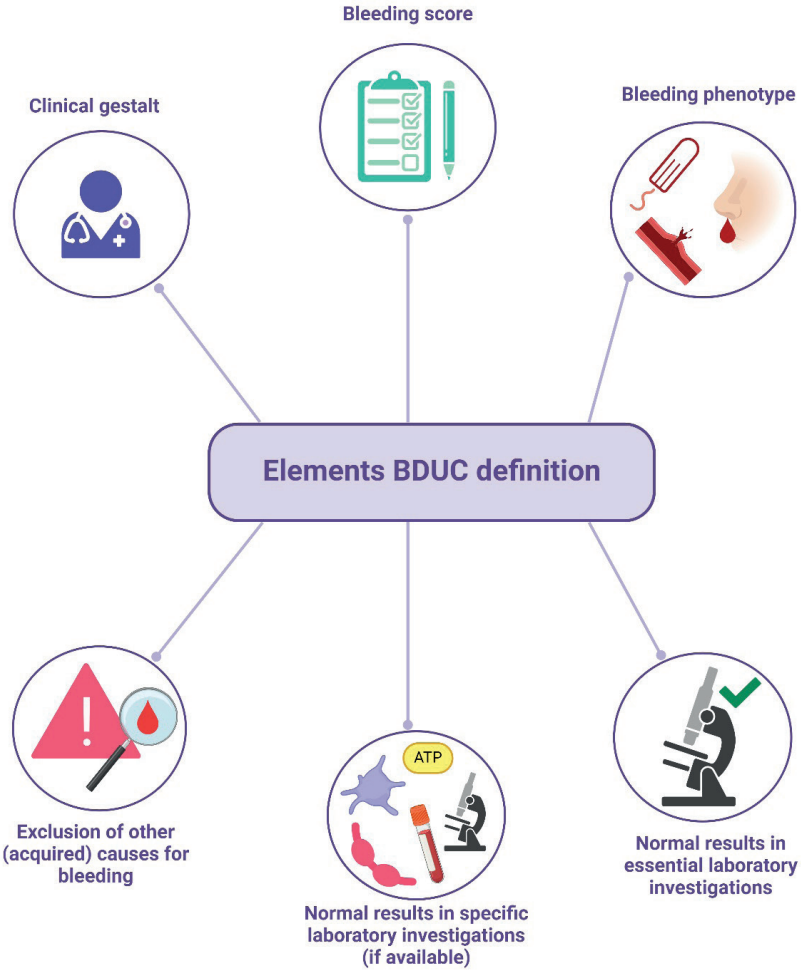


3. Elements of the BDUC definition

Previously used definitions applied in literature were heterogeneous, but all comprised two main criteria:

1. There is an increased bleeding tendency based on the clinical view of the physician (clinical gestalt) and/or increased bleeding score assessed by a validated bleeding assessment tool (BAT)
2. There are no abnormal results from available hemostasis laboratory tests [4-7]

Recently, the ISTH SSC published a BDUC definition, containing the following elements.[2]

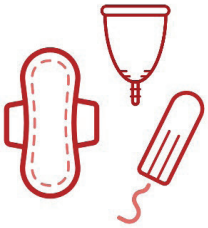


4. Bleeding phenotype

The bleeding phenotype of individuals with BDUC is typically characterized by mucocutaneous bleeding as well as bleeding around medical and dental procedures. [3-5,9,10]

Gynecologic & obstetric bleeding

Heavy menstrual bleeding



60% - 90% of females with BDUC [3-5, 10]

Gynecologic cause should be excluded [2]

Post partum bleeding



30% - 65% of females with BDUC [3-5,9,10]

Mucocutaneous bleeding

Hematomas/easy bruising



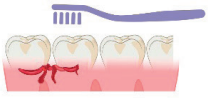
66% - 78% [3-5, 10]

Epistaxis



31% - 79% [3-5, 10]

Oral mucosal



19% - 53% [3,4,10]

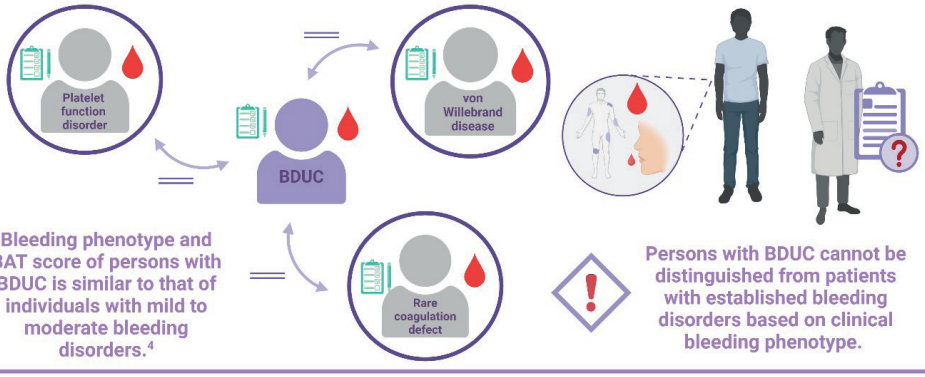
Bleeding during or after medical & dental procedures



44% - 75% [3-5,9,10]

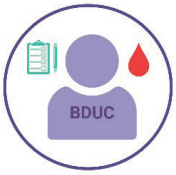


36% - 84% [3-5,10]



5. Assessment of bleeding phenotype

Phenotypic assessment of bleeding is central to the diagnosis of BDUC



How to quantify the bleeding symptoms?

1

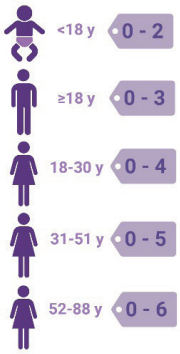
Bleeding Assessment Tool (BAT)

BATs provide a standardized and objective approach for the assessment of bleeding symptoms.

The International Society on Thrombosis and Haemostasis (ISTH) BAT is currently the most applied BAT and the recommended assessment tool by the ISTH SSC.[2,11]

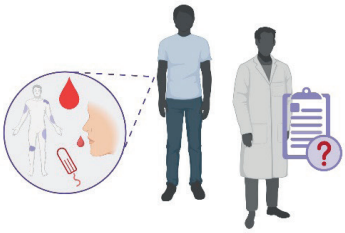
Reference ranges ISTH-BAT

Adjusted reference ranges have been recommended based on age.[2, 14]



2

Clinical gestalt assessed by medical specialist



BATs have limitations including: [2, 12]

- Lack of sensitivity in persons without hemostatic challenges
- Recall bias
- Score saturation with recurrent symptoms
- Inability to differentiate between different types of MBD.

Moreover, low BAT scores do not always exclude mild bleeding disorders.[13]

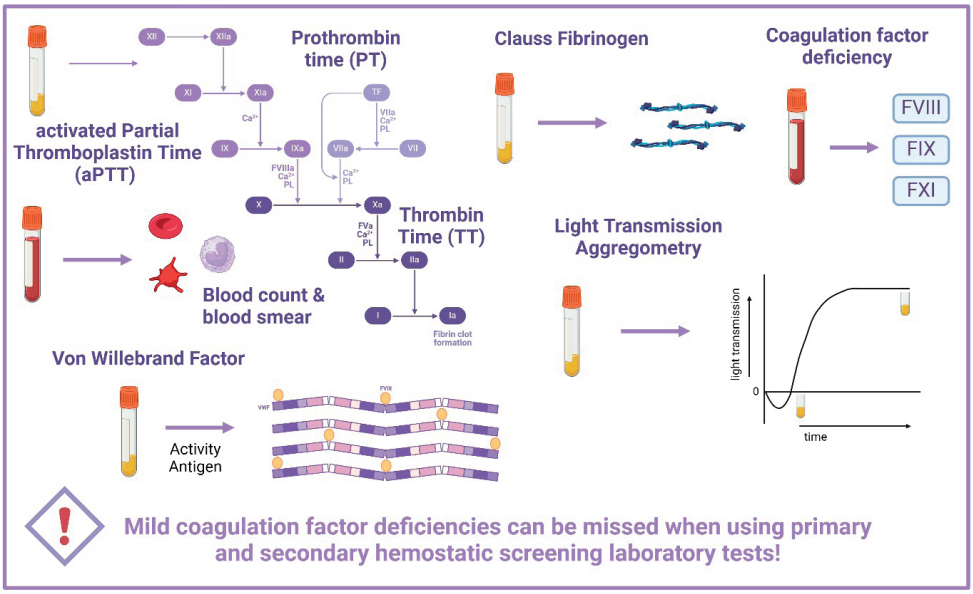


As BATs have several limitations, BAT scores need to be considered on an individual basis together with the clinical gestalt by the treating physician.

6. Laboratory tests

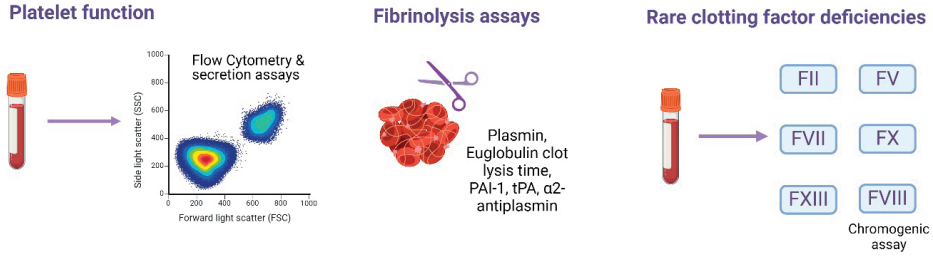
Laboratory testing is indispensable in the diagnostic process of bleeding disorders. Various diagnostic algorithms for laboratory testing leading to BDUC diagnosis have been described. Recently, the ISTH SSC recommended a stepwise approach.[2] In addition, other causes for bleeding symptoms should be excluded.[15] In case of a clinically relevant bleeding tendency without any abnormal laboratory test results, BDUC may be diagnosed.

1 Essential Laboratory Investigations



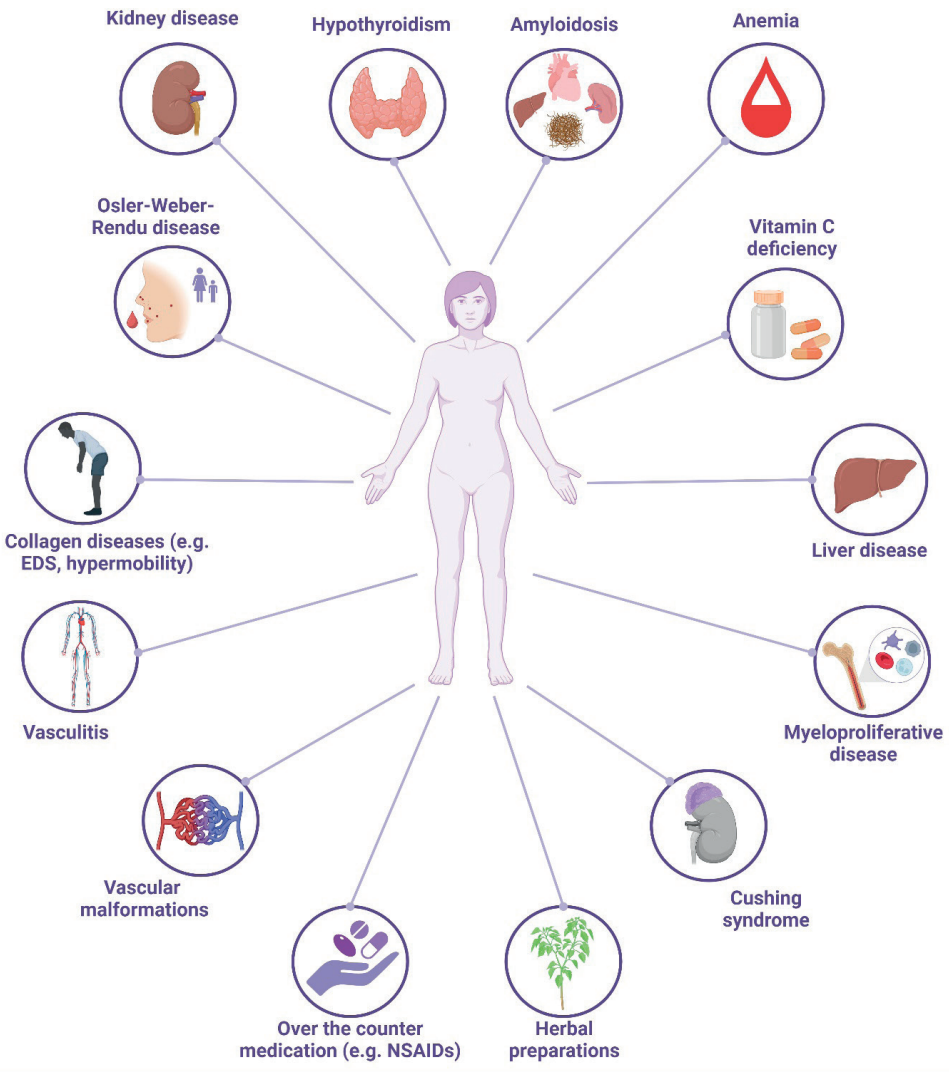
2 Specific Laboratory Investigations (if available)

Additional laboratory tests can be performed if available. These investigations can include platelet assays, fibrinolysis assays, rare clotting factor deficiencies and other specialized assays.[2]



7. Other causes for bleeding

Other conditions are associated with an increased bleeding tendency, without detectable abnormalities in the coagulation cascade.[15] To exclude other causes, additional (laboratory) tests should be performed when clinically indicated.



8. Current treatment

Indications for treatment



Bleeding e.g. heavy menstrual bleeding



Dental procedures



Surgeries and other medical interventions



Pregnancy and delivery



Persons with BDUC have an increased bleeding risk around hemostatic challenges!



Regularly, a treatment plan is composed for these circumstances.[15]



Clear guidelines on treatment in persons with BDUC are lacking.

Currently used treatments

A step-wise treatment plan is suggested for persons with BDUC, based on previous bleeding complications and severity of the bleeding tendency: [2,15]

Tranexamic acid (TxA)



Minor and major medical and dental procedures



- 1g 3x/day [15]
- 15-25 mg/kg (up to 1g) 3x/day [5]
- 500-1000 mg 3x/day [16]



Single dose 10 mg/kg (up to 1g) continued orally [16]

Desmopressin (DDAVP)



Minor and major medical and dental procedures, often combined with TxA



0.3 µg/kg [5, 15, 16]

Platelet transfusions



Preventative and/or therapeutic in high risk situations

* Consider risk on alloimmunization!



Some case studies report the use of fresh frozen plasma in persons with BDUC.[17]

Recombinant activated FVII



Last resort in case of major, life threatening bleeding complications

Treatment knowledge gaps

Only few studies have investigated treatment and bleeding complications around hemostatic challenges in persons with BDUC.[5, 9, 16] Consequently, there is little evidence regarding efficacy and safety, and unknowns remain.

Best treatment per medical intervention



Efficacy



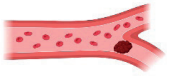
Bleeding complications



Safety



Thrombotic risk

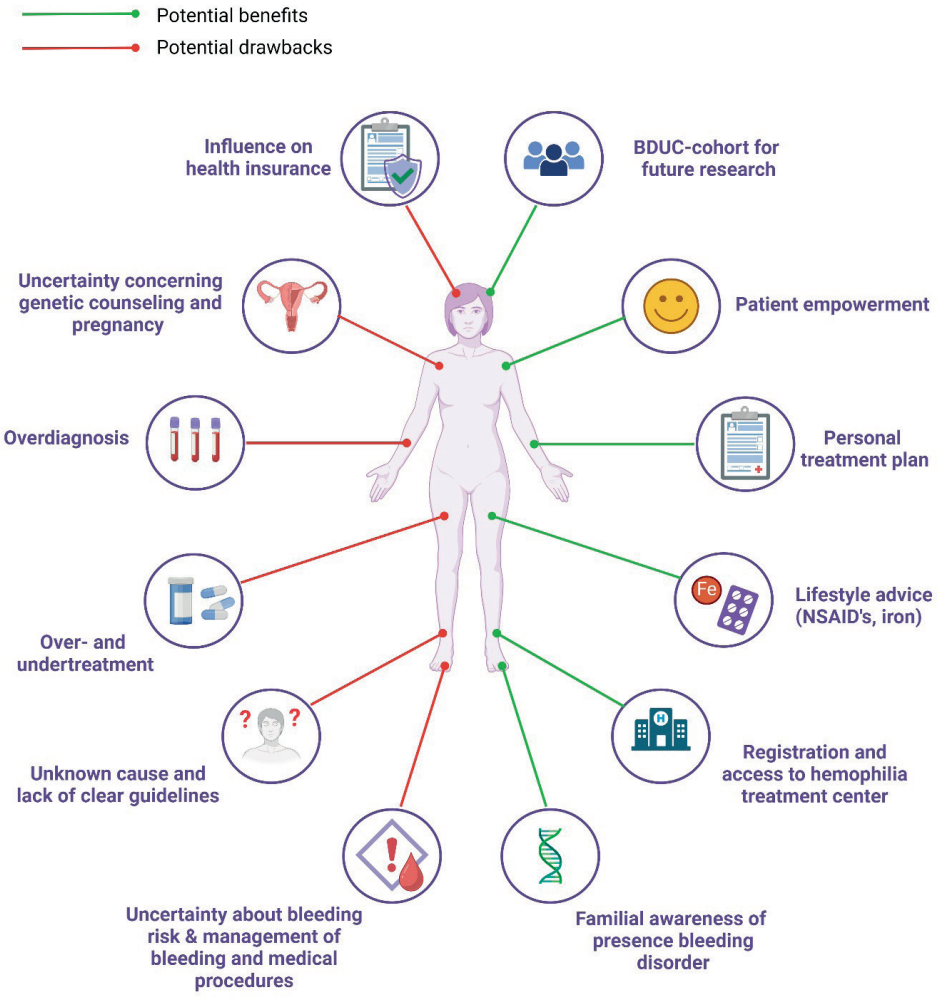




Bleeding complications still occur which suggests that the currently applied prophylactic treatment in BDUC is often not adequate.[9]

9. Benefits and drawbacks of BDUC diagnosis

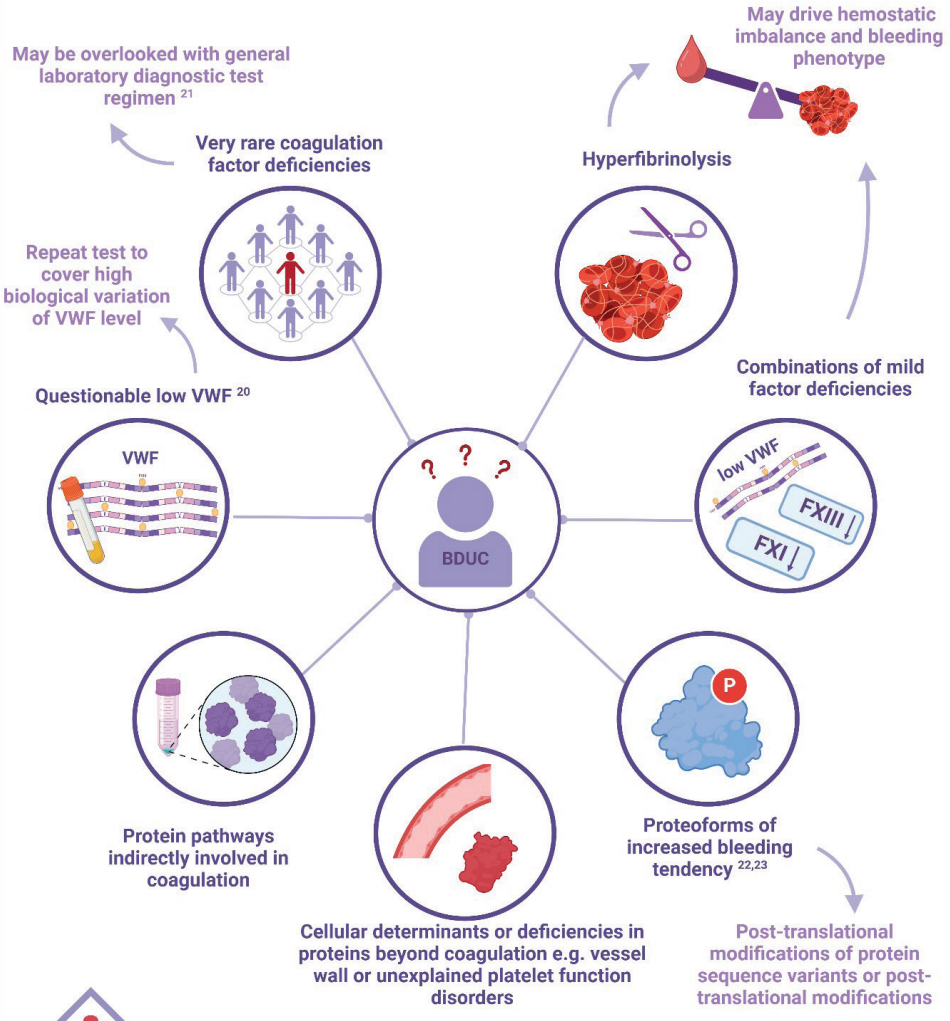
As the bleeding phenotype of BDUC patients is similar to patients with other bleeding disorders, it is not surprising that BDUC patients also present with an increased morbidity and lower quality of life. The diagnosis of BDUC has various positive and negative implications.[7,15,18]



Adapted from Baker et al. 2021

10. Suspected hemostatic etiologies underlying BDUC

A variety of pathologies can be associated with an increased bleeding tendency or prolonged bleeding episodes.[19, 20] The underlying pathophysiology behind the bleeding tendency in persons with BDUC is yet unknown.



BDUC most likely comprises a heterogeneous group of different underlying defects causing bleeding symptoms.

11. Advanced hemostatic laboratory testing

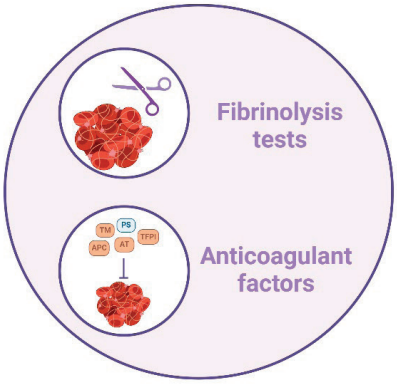
Besides the broadly available hemostatic laboratory tests, additionally more advanced hemostatic laboratory tests can be used to investigate underlying pathophysiological mechanisms.



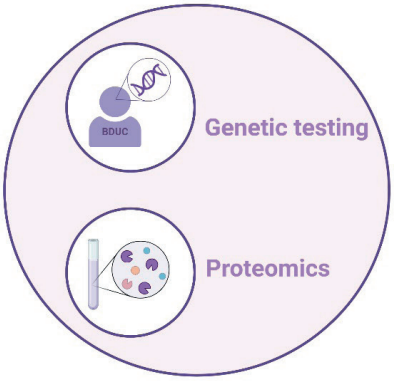
These tests are currently mainly used in research settings and their diagnostic value in the BDUC population is unclear.



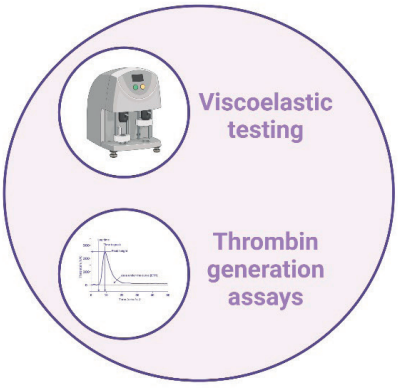
Functional testing



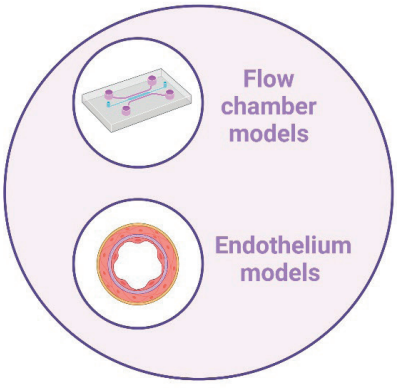
Genomics and proteomics



Global hemostasis



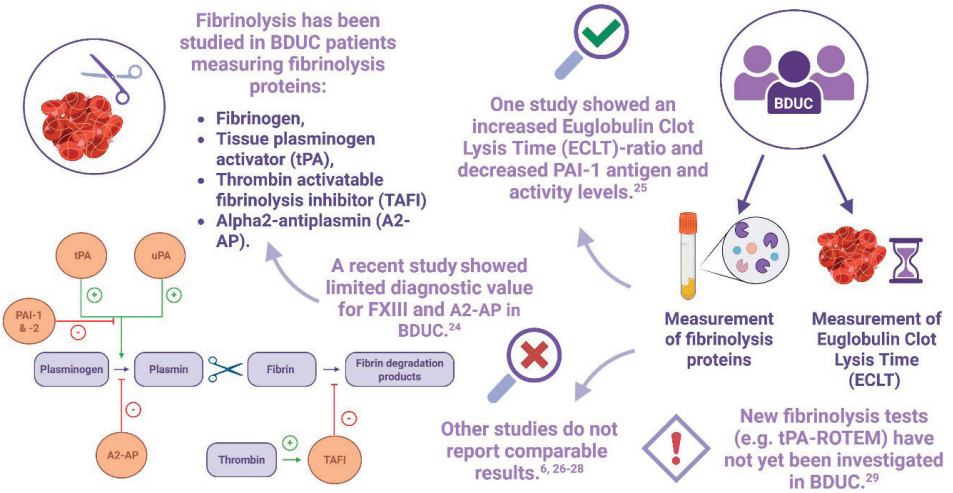
Microfluidics



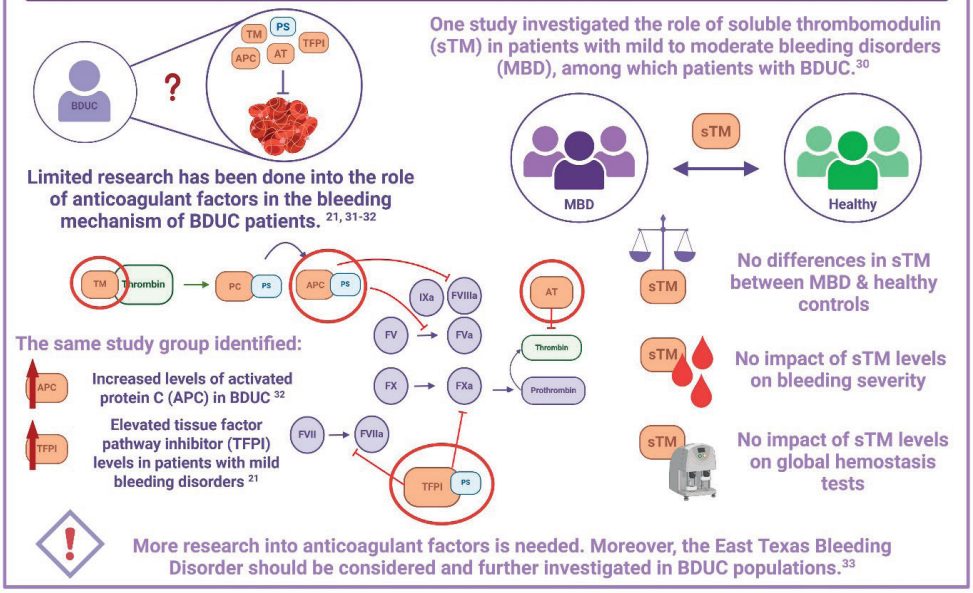
In the next four capsules current research and knowledge gaps on advanced hemostatic laboratory testing in BDUC are highlighted.

12. Functional testing

Fibrinolysis tests



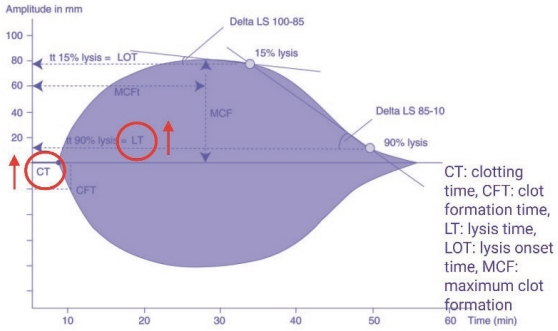
Anticoagulant factors



13. Global hemostasis

Viscoelastic testing





CT: clotting time, CFT: clot formation time, LT: lysis time, LOT: lysis onset time, MCF: maximum clot formation

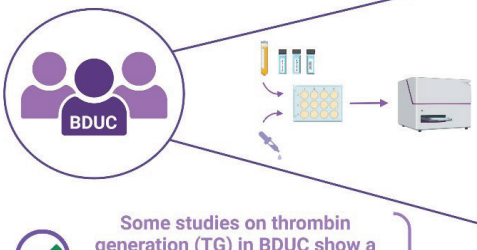
Viscoelastic testing using rotational thromboelastography (ROTEM®) in BDUC patients has not shown any abnormalities.[28]

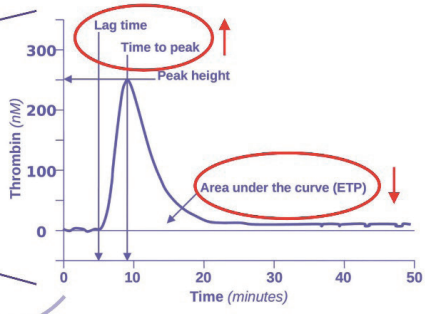
In 9% of the patients in this cohort, NATEM showed (mildly) prolonged clot time and maximum lysis.

Only in the **NATEM-assay**, in which whole blood is recalcified without addition of other activators, mild deviations were seen in a small part of the study population.[5]

Evaluation of the NATEM-assay in larger cohorts may provide insight in the value of the previously found abnormalities.

Thrombin generation assays





Some studies on thrombin generation (TG) in BDUC show a prolonged lag time and time to peak and/or a diminished maximal thrombin generation.[5,6,28]

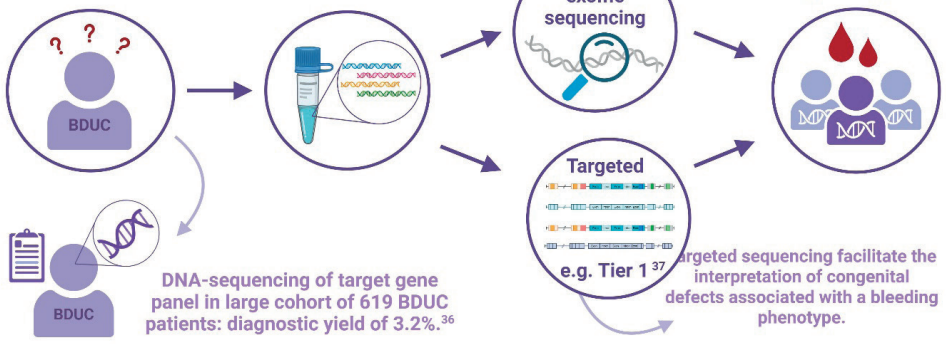
However, other studies do not confirm these findings.[26,34]

Discrepancies in test results may be explained by the heterogeneity of test methods.[1] Novel TG assays specifically designed for bleeding evaluation need to be investigated in BDUC.

14. Genomics & proteomics

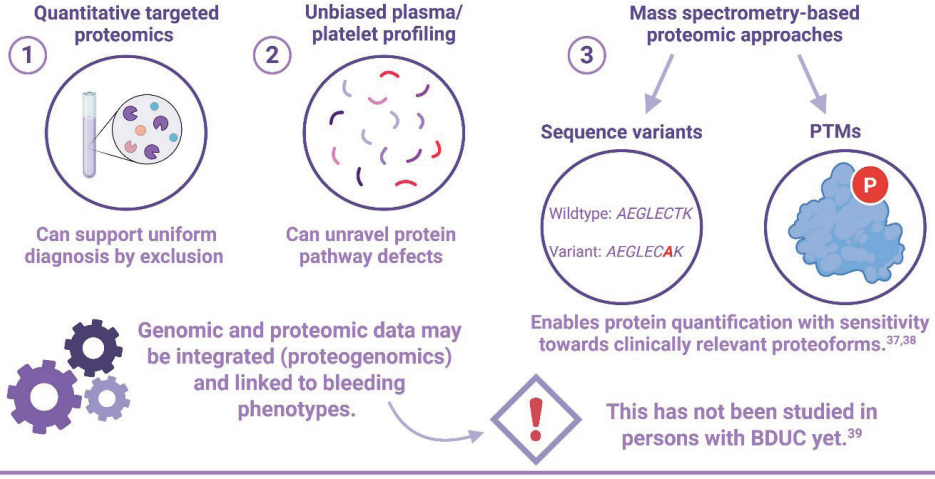
Genomics

The etiology underlying BDUC may be caused by a genetic abnormality. Exome variants resulting in altered protein biosynthesis may affect protein functionality within hemostasis.



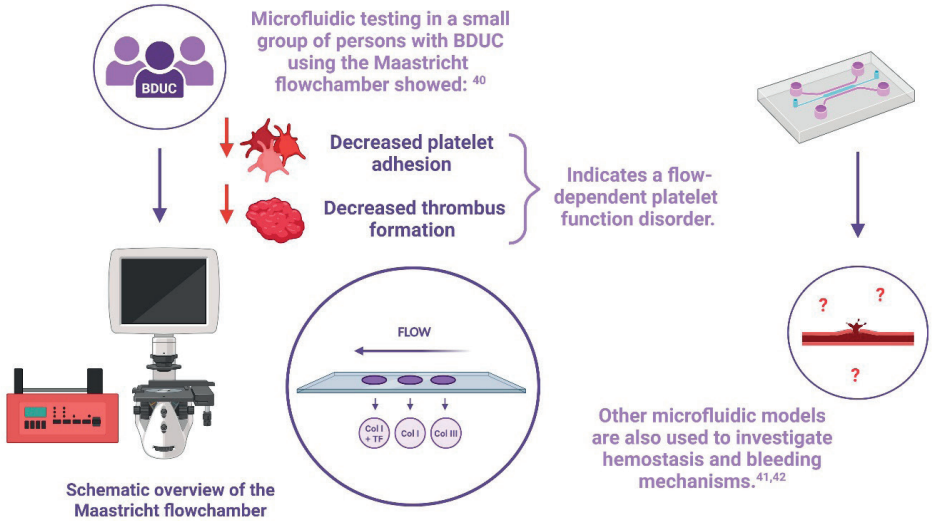
Proteomics

Powerful strategy to screen for rare protein deficiencies or abnormal protein signatures.

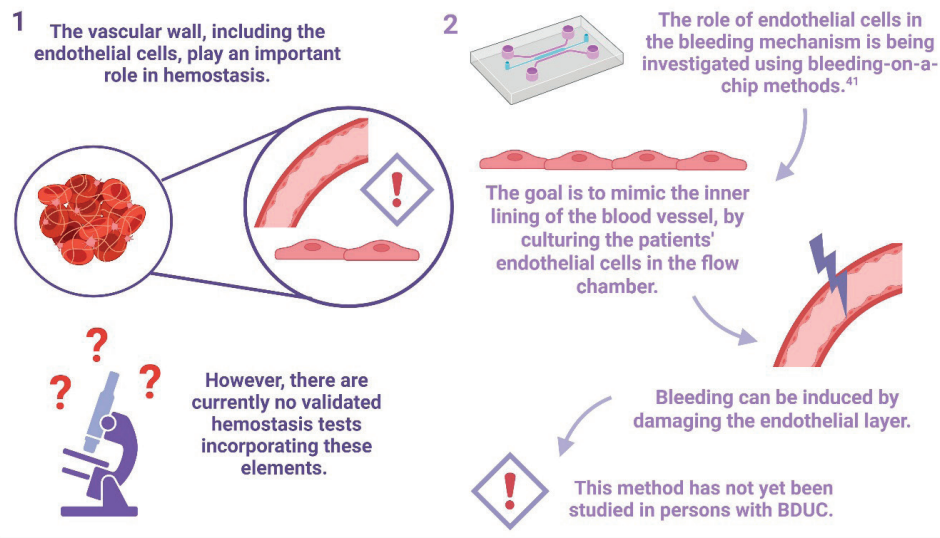


15. Microfluidics

Flow chamber models



Endothelium models



16. Quality of life

Patient-reported outcomes measurement information system (PROMIS)

Tool to precisely and efficiently measure:



Patient reported symptoms



Functioning



Health-related quality of life

Consisting of person-centered measures that evaluates and monitors: [43]



Physical health



Social health



Mental health



General population



Chronic conditions

Validation of PROMIS

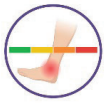
PROMIS items validated in hemophilia patients: [44]



Hemophilia



Physical function



Pain interference



Fatigue



Depression



Anxiety



Ability to participate in social roles & activities



Satisfaction with social roles and activities

PROMIS reliable and useful instrument to measure patient-reported outcomes in hemophilia.



BDUC

- Limited research has been performed into quality of life and/or PROMIS in BDUC.
- One recent paper by Mehic et al. reported impaired physical and mental health-related quality of life in persons with BDUC using the RAND-36.[45]
- The Reliability of PROMIS to measure PROs in BDUC is unknown.

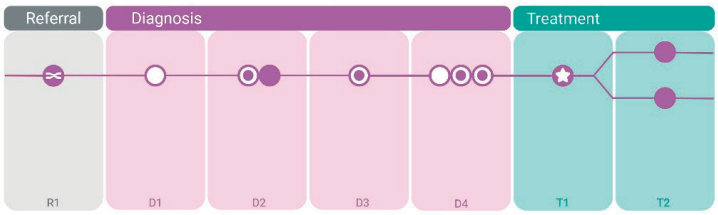
17. The BDUC health journey

Identifying care pathways and patient experiences are essential to generate meaningful insights into the perceived challenges and areas for improvement in the currently provided care.

Identifying the BDUC care pathway

A care pathway is a visual display of the entire care process and gives insight into:

- Fixed contact moments between the health care provider and the patient
- Timing of care
- Responsibilities during the entire care process



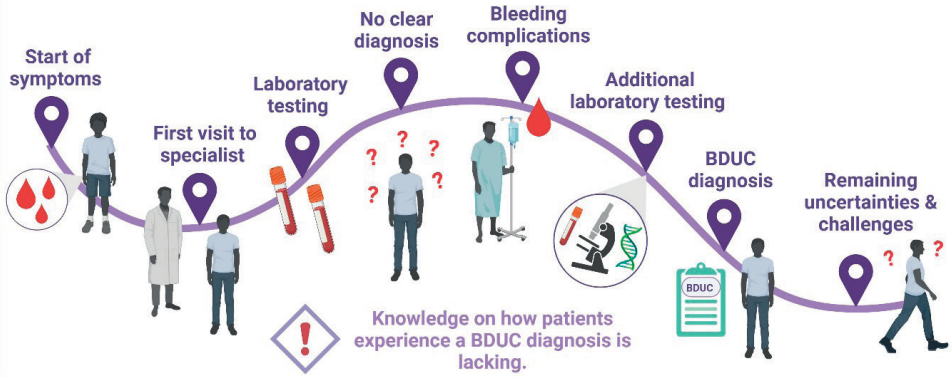
The care pathway for persons with BDUC has not been identified yet.

Visualizing the care pathway makes it possible to (better) organise & standardize care: [46-50]

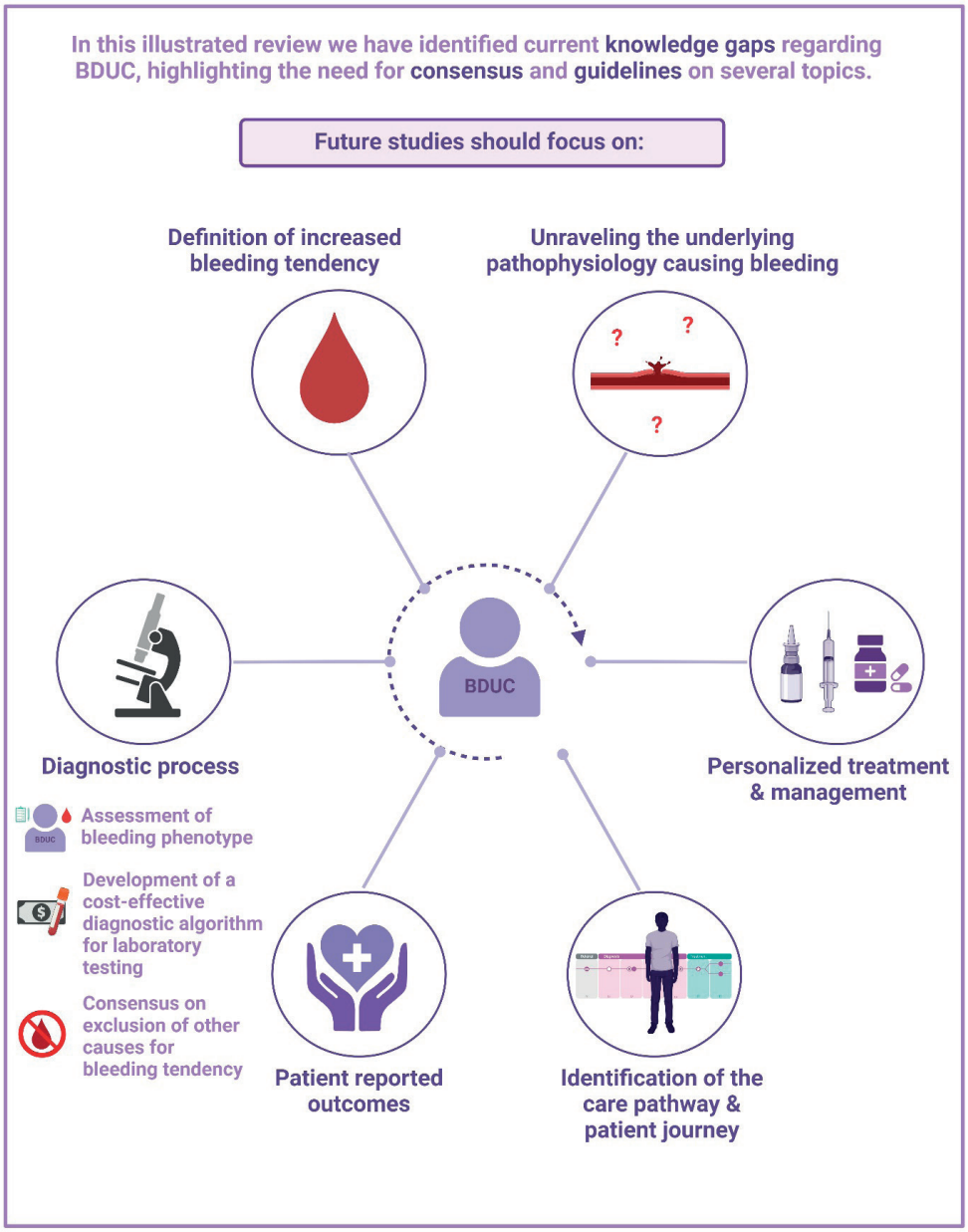
- Improves patient outcomes
- Improves quality of care
- Supports communication
- Improves compliance to guidelines
- Reduces costs

The BDUC patient journey

To enable patient-centered care and facilitate the delivery of the best possible healthcare experiences, it is essential to gain insight into patient experiences with the disease involved and provided care, and their associated (unmet) needs.



18. Future directions of BDUC research



19. Key takeaways

1 In half of individuals referred for a bleeding tendency analysis a clear diagnosis cannot be made. This is then referred to as an unexplained bleeding tendency or 'bleeding disorder of unknown cause' (BDUC).



2 Identified knowledge gaps & future research topics:

I. Definition of increased bleeding tendency

II. Pathophysiological mechanism

III. Diagnostic process

IV. Treatment & management

V. Patient reported outcomes

VI. Care pathway & patient journey

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CHAPTER 3

CURRENT PRACTICE REGARDING BLEEDING DISORDER OF UNKNOWN CAUSE IN THE NETHERLANDS

a national survey

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ABSTRACT

Background: About 40-70% of persons with a clinically relevant bleeding tendency who are referred to hemostasis experts are classified as having a 'bleeding disorder of unknown cause' (BDUC) as no biological entity can be found after extensive laboratory testing. Currently, guidelines are under development regarding diagnostic assessment and management to minimize variation in clinical practice.

Aim: To investigate current practices regarding BDUC in the Netherlands.

Methods: An online survey on the best BDUC definition, associated bleeding phenotype, clinical and diagnostic approaches, treatment, registration, and follow-up was distributed amongst healthcare providers working in Dutch hemophilia treatment centers (HTCs).

Results: The survey was completed by 39/54 (72%) respondents. Twenty percent did not register BDUC patients in their HTC. Healthcare professionals indicated that follow-up should depend on bleeding phenotype severity and bleeding history, and other potential causes for an increased bleeding tendency should be excluded. Moreover, the use of laboratory tests within the routine diagnostic pathway was demonstrated to be heterogeneous. Regarding treatment, tranexamic acid was most frequently prescribed for minor and major surgical interventions (79% and 86%), dental extractions (93%) and childbirth (93%). Desmopressin was prescribed for major surgical procedures by 79%.

Conclusion: Our survey shows that Dutch current practice varies, but is generally in line with recent ISTH SSC recommendations. Additionally, it describes other clinically relevant topics not included in the international survey such as follow-up and exclusion of other causes for bleeding. This survey therefore adds to international efforts to unify BDUC definition, diagnostic approach, treatment and follow-up, and to attain broadly supported guidelines.

INTRODUCTION

In about 40-70% of persons who are referred to a hemostasis expert with a clinically relevant bleeding tendency, no biological entity can be found after extensive laboratory testing (1, 2). These persons are classified as having a bleeding disorder of unknown cause (BDUC). Most common bleeding symptoms in persons with BDUC are mucocutaneous bleeding, especially heavy menstrual bleeding and post-partum bleeding, and bleeding after medical and/or dental procedures (3-5). The bleeding phenotype of persons with BDUC, often quantified using a bleeding assessment tool (BAT) score, is quite comparable to that of individuals with moderate to mild inborn bleeding disorders such as Von Willebrand disease (VWD), mild coagulation factor deficiencies and less severe platelet function disorders (5). Therefore, the bleeding pattern of persons with BDUC hardly discriminates itself from other established bleeding disorders. Currently, guidelines are under development and urgently needed as clinical management including treatment and follow-up, and laboratory testing for diagnosis varies significantly.

In 2024, the BDUC Scientific and Standardization Committee (SSC) of the International Society of Thrombosis and Haemostasis (ISTH) published a short list of recommendations to standardize BDUC definition and clinical management of persons with BDUC, including the minimal standards for laboratory testing, treatment strategies and the importance of registration of persons with BDUC based on a worldwide survey (2). Nevertheless, some clinical aspects of BDUC healthcare were not addressed and knowledge gaps and challenges remain, especially as underlying pathophysiological mechanisms of bleeding are still unknown. With this study, the Bleeding Disorder of Unknown Cause in the Netherlands (BDUC-iN) working group aims to add to current knowledge of clinical practice by more broadly investigating BDUC approaches in the Netherlands.

MATERIALS AND METHODS

A survey was designed consisting of four open questions and 25 5-point Likert scale questions, ranging from 1 (strongly disagree) to 5 (strongly agree) for statements, and from 1 (never) to 5 (always) for frequencies. Questions covered the BDUC definition, associated bleeding phenotype, diagnostic and clinical approaches, applied treatment regimens, registration and follow-up of persons with BDUC. The questionnaire was designed in concept by C. Mussert and A. Monard and complemented and refined by all members of the BDUC-iN working group. The survey was performed within the online survey and data system SurveyMonkey (6). On June 8th, 2023, this survey was sent to all hematologists (pediatric and adult), internists vascular medicine, nurse practitioners and clinical chemists, working in one of the six Dutch hemophilia treatment centers (HTCs), which

comprised 54 recipients in total. Possible answers included the option “not applicable” that could be filled in by respondents if they considered themselves lacking the required expertise to answer a specific question. A reminder was sent out every six weeks until the survey was closed on October 6th, 2023. All respondents gave consent to use the collected data for research purposes.

Not all respondents filled in every question, causing missing data. Results were expressed in percentages together with the number of collected responses. Not applicable answers were excluded from the numerator. Answers from laboratory specialists were excluded for clinically orientated questions, which includes the observed bleeding phenotype, other causes for bleeding, registration, follow-up and treatment strategies.

Software IBM SPSS version 28 was used for the descriptive statistics (frequencies in percentages and numbers). Agreement was defined as ≥70% rating a statement as “strongly agree/agree” or “strongly disagree/disagree”, likewise with regard to frequencies. Subgroup analyses were performed comparing physicians with nurse practitioners and comparing respondents working with pediatric patients and adult patients.

RESULTS AND DISCUSSION

In total 39/54 (72%) recipients from six HTCs completed the survey among which 24 (62%) physicians, 8 (21%) nurse practitioners and 7 (18%) clinical chemists. 13/38 (34%) respondents worked with children, 15/38 (39%) worked with adults and 10/38 (26%) worked with both. The mean years of experience regarding bleeding disorders was 15 years, ranging from 1 to 33 years.

BDUC definition

Respondents emphasized the following elements should be included in the BDUC definition: (i) presence of an increased bleeding tendency (100%), (ii) exclusion of other bleeding disorders (95%), (iii) exclusion of acquired causes for an increased bleeding tendency (90%), (iv) absence of abnormal laboratory test results (85%), (v) presence of an elevated BAT score (77%), and (vi) specification of laboratory tests to perform (71%). Only fifty seven percent of respondents indicated that bleeding phenotype assessment based on clinical gestalt should be an element in the BDUC definition, however in a follow-up question on what diagnostic instruments should be used to score bleeding tendency severity, 87% indicated the use of clinical gestalt to assess bleeding phenotype.

These elements were in line with the published ISTH SSC BDUC definition, stating that these are patients with a high suspicion of a bleeding disorder based on medical history and without abnormal test results in a specified set of laboratory tests (2).

Bleeding phenotype

In line with the ISTH SSC communication and previous research (2-5, 7), the types of bleeding reported by our respondents, categorized as "often" or "always" observed in persons with BDUC, were: menorrhagia (94%), cutaneous bleeding (90%), and bleeding after surgical (77%) and/or dental procedures (68%). Central nervous system (CNS) bleeding, hematuria, muscle and joint bleeding were reported to be "never" or "rarely" seen by 70%, 71%, 81% and 84% of respondents respectively.

Of 31 respondents, 81% "often" or "always" observed a mild bleeding phenotype, according to EHA definitions (8), in persons with BDUC. A moderate bleeding phenotype was "often" or "always" observed by 42% and "sometimes" by 55% of respondents. A severe phenotype was only observed "sometimes" by 48% and "never" or "rarely" by 45%.

Ninety percent (27/30) of respondents confirmed the use of a BAT, with the ISTH-BAT being the most frequently used BAT (89%).

Laboratory testing

Since BDUC is a diagnosis by exclusion, the use of laboratory tests is essential in the diagnostic pathway. Figure 1 shows which laboratory tests are performed, only performed on specific indication or not performed in the Netherlands. Laboratory tests indicated to be performed by ≥70% of respondents were CBC, PT, aPTT, vWF activity, FVIII, vWF antigen, fibrinogen, MPV, FIX, FXIII, LTA and PFA-200. ≥70% reported not to perform plasma clot formation, TAFI and plasma clot lysis under pressure. No clear consensus was found regarding the use of the remaining laboratory tests.

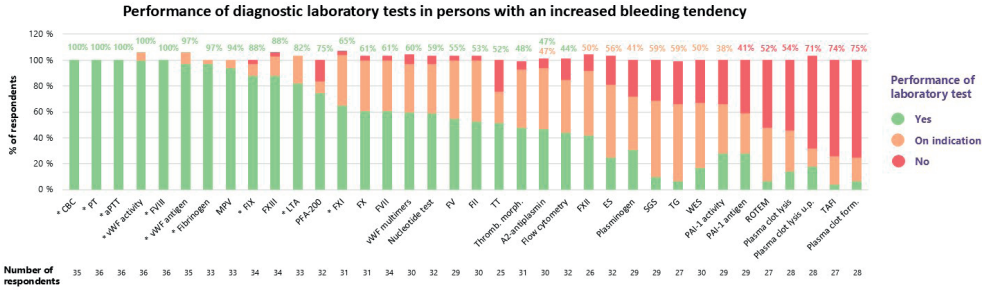
Overall, tests that were indicated to be performed in our study were comparable to the laboratory tests in the proposed standard panel in ISTH SSC recommendations (2). A difference was found regarding the measurement of FXI which is recommended to standard perform by the ISTH, whilst in our survey consensus on FXI measurement was nearly reached (65%). Another difference was the consensus in our survey to also perform PFA-200. The use of the PFA-200 in BDUC is under debate. It was not recommended by the ISTH SSC, although several studies have shown unexplained PFA-200 prolongations in BDUC patients (9, 10). Furthermore, within the international ISTH SSC survey platelet function tests were remarkably much less frequently performed than indicated by our respondents (11). This was especially the case for nucleotide tests and/or flow cytometry in addition to LTA testing. The ISTH SSC recommends to perform these additional

platelet function tests if available, but they are not mandatory for a BDUC diagnosis (2). Although LTA is required for a BDUC diagnosis according to the ISTH SSC, only 82% of our respondents indicated to perform this test (some of them only on indication). These results suggest that some HTCs perform extensive platelet function tests, whilst in others there is still room for improvement regarding platelet function diagnostics. A possible explanation for the higher nucleotide tests use in the Netherlands could be the higher availability of these specialized tests in Dutch HTCs compared to a more limited availability in other (less developed) countries. Apart from its availability, the high workload and the need of specialized analysts are also important limitations of these tests. Despite, Dutch healthcare providers seem to agree that it is relevant to perform these tests in patients with a suspicion for a (primary) hemostasis disorder who would otherwise be classified as BDUC.

Tests that were mainly reported to be "never performed" or only "on indication" were more advanced hemostatic laboratory tests such as ROTEM, thrombin generation and fibrinolysis tests, which are often used to investigate underlying pathophysiological mechanisms in research settings and are not broadly available in all HTCs. Moreover, these specialized assays give variable results (4, 12-18). Therefore, their added value in the diagnosis assessment for BDUC is still unclear.

Subgroup analyses were performed to investigate possible differences in the diagnostic approach between treating physicians working with children and adults. There was a remarkable difference in the use of platelet function tests, showing that LTA and nucleotide tests were more often performed 'on indication' in children compared to adults instead of standard measurement. Alternatively, flow cytometry was more often performed in children than adults. This could be explained by the fact that LTA and nucleotide tests require larger blood volumes, which is not always feasible in (small) children, whilst flow cytometry requires little volumes and is therefore easier to perform in children. Another difference was seen in the use of fibrinolytic tests, including plasminogen, α2-anitplasmin, PAI-1 antigen, plasma clot lysis and plasma clot formation, showing that these test were mainly performed 'on indication' in children, whilst they were more often performed 'standard' in adults.

As BDUC is a diagnosis of exclusion the question remains however what we consider normal investigations, whether a BDUC diagnosis is only based on the reference range of laboratory test results or whether (a combination of) low normal test results could also be an explanation for an increased bleeding tendency.



Abbreviations: CBC: Complete Blood Count; PT: Prothrombin Time; aPTT: activated Partial Thromboplastin Time; vWF: von Willebrand Factor; MPV: Mean Platelet Volume; TT: Thrombin Time; LTA: Light Transmission Aggretometry; PFA: Platelet Function analyzer; TG: Thrombin Generation; SGS: Single Gene Sequencing; ES: Exome Sequencing (TIER-1 genes); WES: Whole Exome Sequencing; Thromb. Morph: Thrombocyte morphology; PAI: Plasminogen Activator Inhibitor; Plasma clot form.: Plasma clot formation; TAFI: Thrombin Activatable Fibrinolysis Inhibitor; Plasma clot lysis u.p.: Plasma clot formation under pressure; ROTEM: Rotational Thromboelastometry;

Figure 1: Use of diagnostic laboratory tests in persons with an increased bleeding tendency.

Respondents were asked which laboratory tests they perform and in which order (first round, second round or third round of laboratory tests, on indication or never) for the diagnosis in persons with an increased bleeding tendency. Answers from the different rounds were combined to indicate the overall use of a specific test.

Respondents were able to select multiple answers, e.g. that a test is performed within a certain round as well as on indication. Therefore, for some tests the sum exceeds 100%. For each test, the percentage of the most common answer, given by respondents, is shown above the bar.

* Laboratory tests in the proposed standard panel in ISTH SSC recommendations.

Other causes of bleeding

Respondents agreed on the importance of exclusion of the following bleeding disorders; hemophilia A and B, coagulation factor deficiencies, von Willebrand disease, platelet function disorders (based on LTA, nucleotide test and/or flow cytometry test results), acquired bleeding disorders and fibrin/fibrinogen/fibrinolysis disorders (Figure 2A), which is in concordance with the ISTH SSC recommendations. Furthermore, respondents agreed on the exclusion of other causes of bleeding like ITP/TTP, anemia, over the counter drug use, liver disease, herbal preparations, self-inflicted, DIC and renal disease (Figure 2B).

However, respondents highlighted that exclusion of other causes for an increased bleeding tendency is also dependent on information derived by medical history taking, patient interview, and physical examination of the patient. Importantly, ISTH SSC recommendations include a list of laboratory tests that should be performed to exclude known bleeding disorders, and also provides some suggestions to exclude acquired medical conditions that can cause bleeding, including hypermobility disorders, use of medication and dietary supplements, liver and kidney disease, thyroid dysfunction, myeloproliferative disease and inflammatory disease (2, 11). With this survey we have complemented these recommendations to provide a comprehensive overview of other (non-hematological) causes for bleeding.

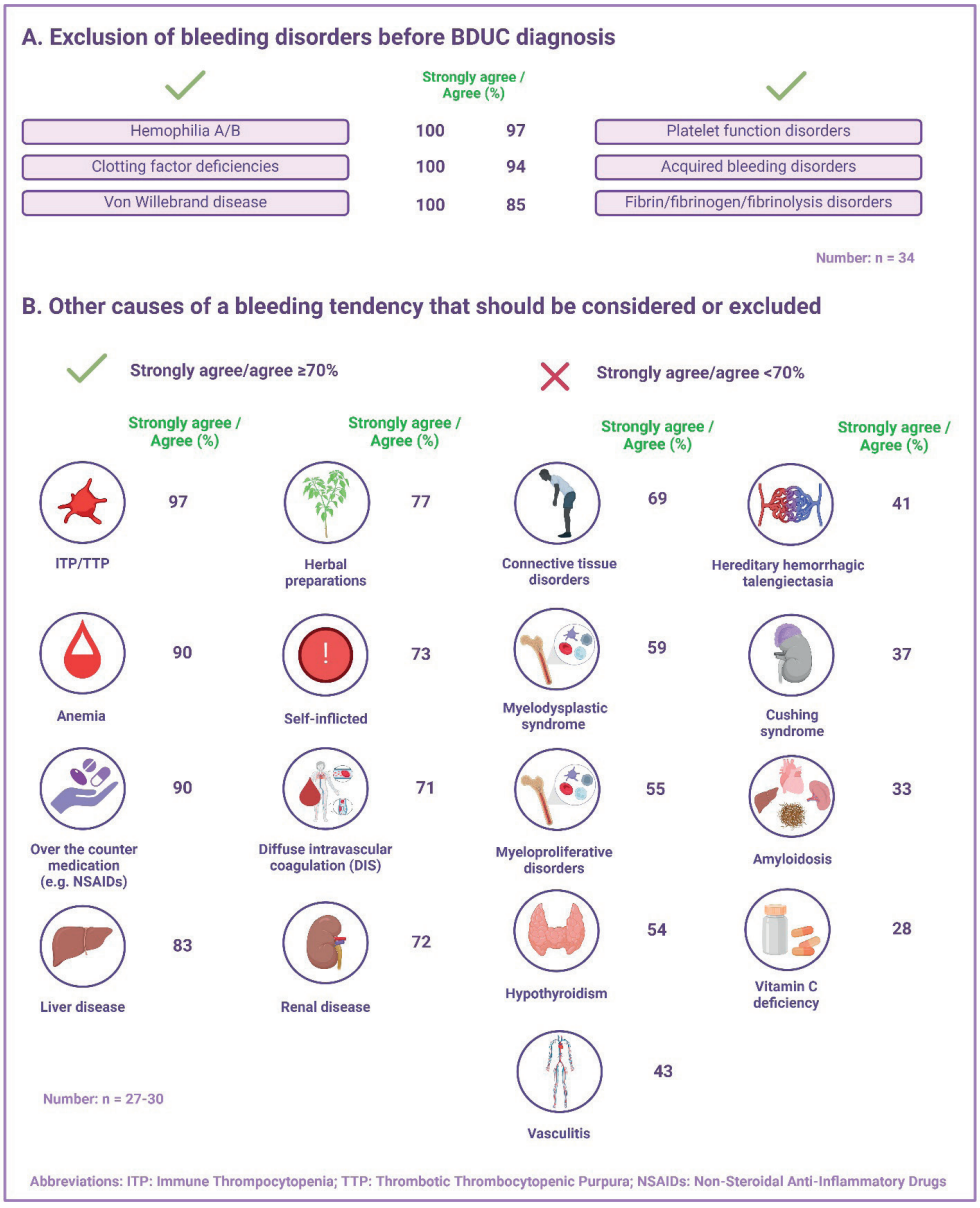


Figure 2: Exclusion of bleeding disorders and other causes for an increased bleeding tendency

Fibrin/fibrinogen/fibrinolysis disorders comprise both quantitative and qualitative fibrinogen defects including hyperfibrinogenemia and (hypo)dysfibrinogenemia, as well as fibrinolytic protein deficiencies and/or abnormalities in global fibrinolysis tests (e.g. euglobulin clot lysis time).

Registration and follow-up

Within our study we identified follow-up frequency (Figure 3) as well as registration. Forty seven percent (14/30) indicated that persons with BDUC are registered as 'bleeding disorder of unknown cause' within their diagnosis registration systems. Sometimes (33%) other nomenclature was used e.g. increased bleeding tendency not otherwise indicated (n.o.i.). Twenty percent of respondents indicated no registration of persons with BDUC at all, possibly causing loss to follow-up. Frequency of registration is in line with the international ISTH SSC survey although we report a higher percentage of registration as BDUC instead of other nomenclature (11).

Regarding follow-up, 76% of respondents indicated that follow-up frequency depends on bleeding severity. Definitions published by the EHA for mild, moderate and severe bleeding phenotype were applied (8). Patients with moderate and mild bleeding phenotypes should receive follow-up care in case of medical interventions or child delivery (both 73% "agree" or "strongly agree"). Moreover, standard follow-up every two years was suggested for moderate and mild bleeding phenotypes by 43% and 37% of respondents respectively, with some suggesting follow-up every 2-5 years. For severe bleeding phenotypes a yearly follow-up was recommended by 53% of respondents. Additionally, 67% "agreed" or "strongly agreed" that follow-up should depend on number of experienced serious bleeding events. The ISTH SSC provided advise concerning (prophylactic) treatment in BDUC patients, but did not incorporate recommendations on follow-up (2).

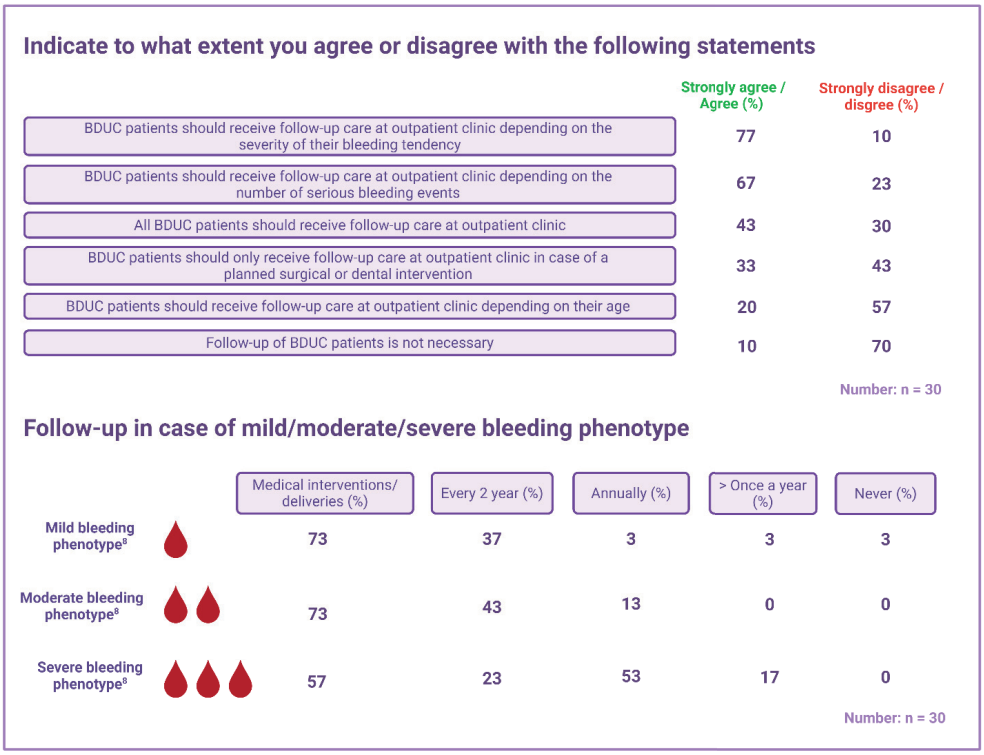


Figure 3: Follow-up prerequisites and frequency in persons with BDUC

Percentages of neutral answers to statements are not shown. Regarding follow-up in case of a mild, moderate or severe bleeding phenotype, the selection of multiple answers was possible.

Treatment strategies

In our study, 97% of respondents “often” or “always” uses tranexamic acid as treatment for persons with BDUC, 59% desmopressin and 17% uses platelet transfusions. Activated recombinant factor VII concentrate was only used “sometimes” by 35% of respondents (Figure 4).

In the Netherlands, persons with BDUC are almost always treated with tranexamic acid when undergoing medical procedures. In case of major surgery, 79% uses or adds desmopressin to the prescribed medication (Figure 4). This is in alignment with ISTH SSC recommendations and although studies on treatment in persons with BDUC are scarce, results also show that tranexamic acid and desmopressin are the most used treatment modalities (4, 19).

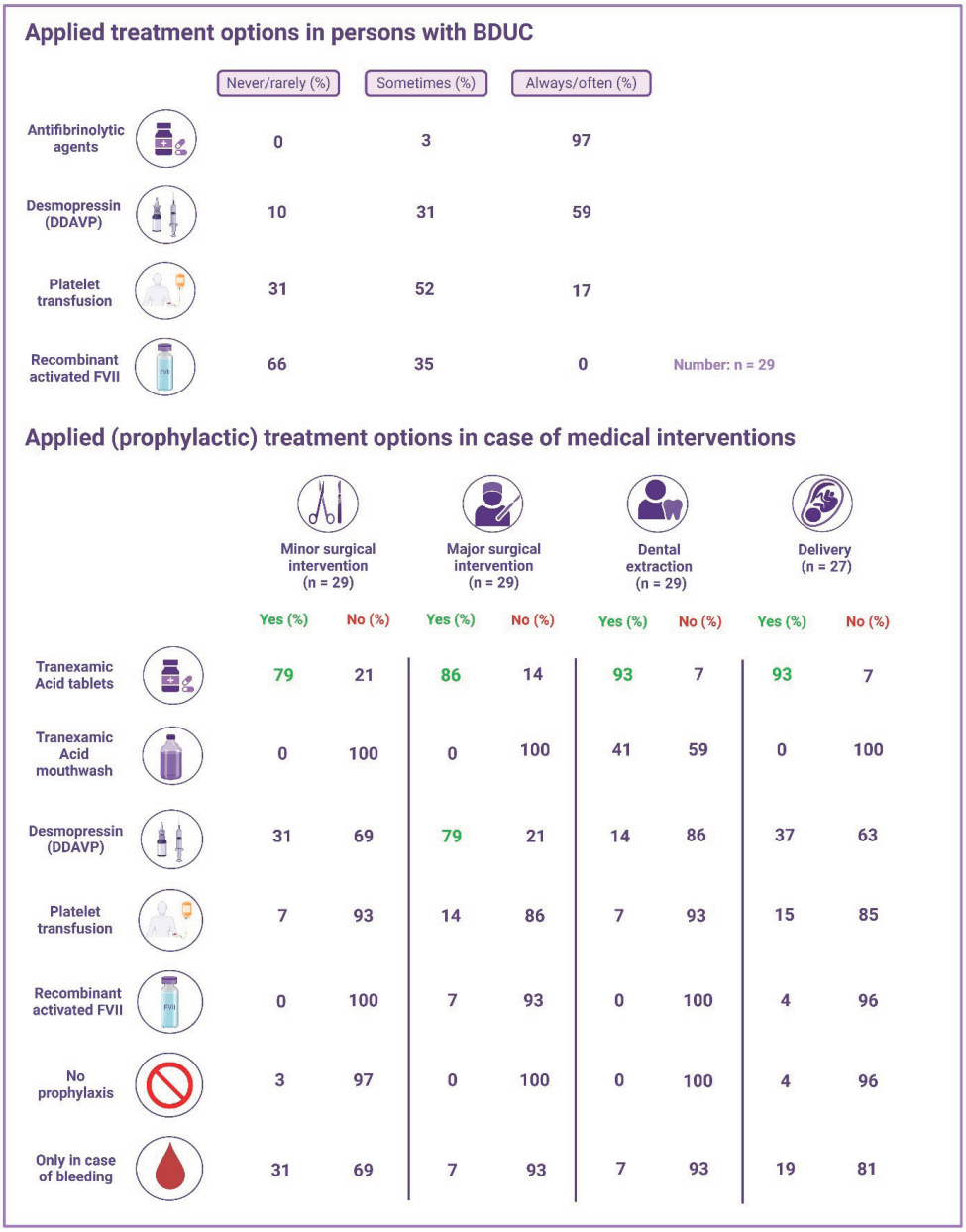


Figure 4: Treatment strategies in persons with BDUC

A selection of multiple answers was possible for (prophylactic) treatment strategies in case of the various medical procedures.

With this survey we have documented current practice in the Netherlands regarding the BDUC definition, diagnostic process and clinical management. Our results align

with an international survey conducted by the ISTH SSC between 2022 and 2023 with a limited number (1-4/216) of Dutch participants (11), although slight differences were found. Moreover, our results showed that not all respondents indicated to use all tests recommended by the ISTH. This could be explained by the former lack of consensus and absent guidelines, but also by differences in test availability across laboratories. Our survey is an important addition to the ISTH SSC recommendations by documenting clinical practice regarding follow-up and the exclusion of a broad range of (hematological) causes of an increased bleeding tendency, as well as highlighting the heterogeneity in the use of more advanced laboratory tests for diagnostic work-up, such as advanced platelet tests, anticoagulant and fibrinolytic factors, specific genetic tests and global hemostasis tests (2). In addition, the inclusion of not only physicians but also specialized nurse practitioners and clinical chemists working with both pediatric and adult populations, ensured a multidisciplinary perspective regarding current practice in BDUC. Nevertheless, to safeguard anonymity respondents were not asked to indicate in which HTC they work, as the number of healthcare providers in Dutch HTCs is limited and answers are easily traced back to the respondent. Therefore, comparisons across HTCs were not possible. Based on the survey results, a widely supported concept BDUC definition was formulated; stating that persons with BDUC have an increased bleeding tendency, based on an elevated (ISTH)-BAT score or clinical gestalt, in whom no abnormalities were found in a specific set of laboratory tests and in whom known bleeding disorders are excluded and other causes for bleeding have been considered.

Our survey has only been distributed to specialists working in hemophilia treatment centers (HTCs), reflecting current practice within highly specialized, mostly Academic centers. Thereby it may not reflect daily practice outside HTCs, where laboratory possibilities are more limited. However, in the Netherlands, persons with BDUC are generally referred to a HTC for extensive diagnostic assessment, so we assume the identified current practice comprises the majority of persons with BDUC.

CONCLUSION

Our survey shows that Dutch current practice is generally in line with ISTH SSC recommendations, although in some cases recommended tests to confirm a BDUC diagnosis are still omitted. Moreover, this survey describes current practice beyond these recommendations. We add that consensus should be established on the exclusion of a broad range of other causes for an increased bleeding tendency, follow-up and the minimal set of laboratory tests that should be conducted. The question remains however whether we should expand the diagnostic pathway for BDUC or use a multi-omics approach and deep clinical phenotyping. Together with ISTH SSC recommendations

this survey therefore adds to international efforts to unify BDUC definition, diagnostic approach, treatment and follow-up, and to attain broadly supported guidelines.

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PART II

PIECING IT TOGETHER:

Functional Testing in the Diagnostic Work-up of BDUC



CHAPTER 4

THE UTILITY OF THE TOTAL THROMBUS-FORMATION ANALYSIS SYSTEM (T-TAS) IN THE THROMBOSIS AND HEMOSTASIS FIELD:

a scoping review

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ABSTRACT

Background: A wide variety of laboratory hemostasis tests is available, but the majority is plasma-based, static and unable to assess platelet function and fibrin formation simultaneously. The Total Thrombus-Formation Analysis System (T-TAS) is a microchip-based flow chamber system that simulates in vivo conditions for evaluating whole blood thrombogenicity.

Aim: A comprehensive overview of its applicability in different thrombosis and hemostasis related clinical situations is lacking and therefore this scoping review was performed.

Methods: A literature search was done using the electronic databases PubMed, Scopus and Embase on January 7, 2024. Original studies assessing the usefulness of the T-TAS in thrombosis and hemostasis related clinical situations were eligible for this scoping review.

Results: A total of 28 studies were included; six studies investigating the role of the T-TAS in congenital bleeding disorders, five studies using the T-TAS to assess 1-year bleeding risk in patients on antiplatelet or anticoagulant medications, four studies investigating the effects of thrombocytopenia and hemodialysis on thrombus formation as measured by the T-TAS, 11 studies testing the applicability of the T-TAS in the monitoring of anticoagulant and antiplatelet therapies and eventually two studies on the ability of the T-TAS to assess the thrombogenicity in different disease entities.

Conclusion: The T-TAS method is an interesting technology that mimics the complex biological coagulation process using shear forces, creating a “blood vessel component on a chip”. More research is needed, but it could eventually function as a screening test for platelet function and coagulation. Moreover, it could be used to detect the presence of anticoagulant and/or antiplatelet medication.

INTRODUCTION

Hemostasis is the process of blood clot formation at the site of vessel injury under arterial or venous flow. It is a complex process consisting of primary and secondary hemostasis and it is regulated by the fibrinolytic system to prevent excessive fibrin formation. Platelet adhesion, platelet activation and platelet aggregation are different mechanisms of primary hemostasis, leading to the initial platelet plug formation. Secondary hemostasis results in formation of thrombin. Activated platelets are able to bind coagulation factors to their procoagulant surface on which the coagulation cascade takes place, resulting in thrombin formation. The formation of thrombin eventually leads to the conversion of fibrinogen to fibrin. Fibrin fibers are able to form cross-linked networks, resulting in a more stable clot (1). Fibrinolysis is the process that degrades the formatted clots by converting fibrin into fibrin degradation products.

A wide variety of laboratory hemostasis tests is available to assess primary and secondary hemostasis. The majority of the tests is performed in plasma, but some (global) hemostasis assays are performed in whole blood. A distinction can be made between screening and confirmatory tests. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) are plasma-based tests used for screening of secondary hemostasis abnormalities in clinical practice, but their sensitivity and specificity is rather low (2, 3). The platelet function analyzer (PFA-200) is a whole-blood test that is used for screening of primary hemostasis function (platelet function and von Willebrand Disease (VWD)), using very high shear rates. It only provides closure times (CT) and normal PFA-200 results do not exclude mild platelet function disorders or VWD (4, 5). Plasmatic tests for quantification of coagulation factor levels are used as confirmatory tests to diagnose congenital and acquired coagulation factor deficiencies and VWD (2, 6). To confirm a platelet function defect, light transmission aggregometry (LTA), performed in platelet rich plasma, is generally considered as the gold standard (7, 8). The main disadvantage of LTA is the labor-intensiveness of the assay and its sensitivity to several pre-analytical conditions (9). Adenosine triphosphate (ATP) release is a test to assess platelet dense granule release (10). Another test for primary hemostasis is the impedance aggregometry (Multiplate), which measures platelet aggregation in whole blood. This test can be useful for diagnosing platelet function disorders (11, 12), for monitoring antiplatelet therapy (13) and/or for predicting the bleeding risk in a perioperative setting (14-16) but lacks the sensitivity of LTA (17). Viscoelastic tests such as ROTEM®, TEG®, ClotPro®, Quantra® and Sonoclot® are whole blood tests introduced for rapid monitoring of plasmatic coagulation, fibrinolysis and platelet count in (massive) bleeding. Dedicated, point of care, whole blood platelet function tests for monitoring antiplatelet therapy such as VerifyNow have been evaluated frequently to detect or exclude the presence of aspirin or P2Y12 inhibitors (18).

Important limitations of the above mentioned tests are the fact that most tests are static, not involving any form of shear, and that they don't take interactions between primary and secondary hemostasis into account. In addition, for the platelet function tests, there is a wide range of assays with different modes of action but poor agreement between the tests (19). Another issue with the currently available platelet function tests is the inability to provide information on platelet function in patients with thrombocytopenia and/or low hematocrit levels, for example in patients with chemotherapy induced thrombocytopenia and bleeding symptoms.

There is a strong need for hemostatic assays that are able to measure both platelet function and fibrin formation simultaneously, in whole blood and incorporating shear in the model, to better depict the in vivo thrombus formation and coagulation process, providing more accurate information by looking at the hemostatic system as a whole.

The Total Thrombus-Formation Analysis System (T-TAS) (Zacros, Fujimori Kogyo Co. Ltd., Tokyo, Japan) is a microchip-based flow chamber system introduced in 2011 by Hosokawa K. et al. (20) (figure 1). This system operates under physiological shear rate and mimics in vivo thrombus formation by measuring platelet function and/or fibrin formation, depending on the chip used, as in physiological hemostasis.

The system is equipped with a pneumatic pump, a flow pressure pump and a rectangular capillary chip. It is an automatic analyzer and claims to provide an easy and rapid quantitative analysis of platelet aggregation, thrombus formation and/or fibrin formation in the microchip under shear flow. By continuous monitoring of variations in flow pressure in the microchip, the thrombus development process can be analyzed (21). This system consists of three types of microchips (platelet chip (PL), atheroma chip (AR) and hemodilution chip (HD)) with different designs and functions that are further explained in table 1.

Approximately 320 µl of whole blood circulates through a capillary channel coated with activators processed by a dedicated computer. As a result, platelets adhere to the thrombogenic surface and form a platelet aggregate with (AR and HD chip) or without (PL) fibrin formation. The formed thrombus leads to an increase in endogenous pressure in the system until the capillaries are completely occluded. The rising pressure of blood and thrombus hardness in the chamber (PL, AR, or HD) reflects the potential of the patient's blood to form a thrombus. T-TAS provides multiple outcome measures including occlusion start time (OST), occlusion time (OT) and area under the curve (AUC).

This scoping review attempts to provide a comprehensive overview of studies of the T-TAS system in the field of thrombosis and hemostasis. In this context, the available information in the literature on T-TAS and its advantages and disadvantages are reviewed.



Figure 1: Picture of the T-TAS 01 system and the three available chips.

METHODS

Protocol

For this scoping review, the following research question was formulated: 'What is known from the existing literature about the T-TAS and its effectiveness in detecting bleeding and thrombotic disorders and the monitoring of anticoagulant therapy?'. The methodological quality of all eligible studies was assessed using the Joanna Briggs Institute (JBI) Appraisal tool. This scoping review was conducted according to the guidelines for scoping reviews by Hilary Arksey and Lisa O'Malley (22).

Literature Search

The literature search was performed using the electronic databases PubMed, Embase, and Scopus. The following search terms were used to identify relevant studies: "total thrombus formation analysis" OR "total thrombus analysis system" OR "T-TAS". The final search date was January 7, 2024. No restrictions concerning language or date were applied. Editorials, commentaries, case reports, reviews and poster or conference abstracts were excluded. All references from eligible studies were checked for other possible relevant titles, until no new titles were found. A flow-diagram of the search strategy can be found in figure 2.

Cartridge	Needed anticoagulant(s)	Sample volume (µL)	Sample stability (for T-TAS 01)	Capillary coated with	Size of capillary	Nr. of capillary channels	Shear rate (S-1)	Indication for use	Assay time
Atheroma (AR)-chip	Citrate* or Hirudin**	450	30 minutes – 3 hours after blood collection	Type I collagen and tissue thromboplastin	300 µm wide × 80 µm deep	1	240 or 600	Designed for quantitative analysis of platelet adhesion & aggregation, granule secretion and thrombus growth	~30 min
Platelet (PL)-chip	Hirudin**BAPA***	320	30 minutes – 6 hours after blood collection	Type I collagen	40 µm wide × 40 µm deep	2	1000 or 1500 or 2000	Designed for assessment of whole blood hemostasis, including primary and secondary hemostatic functions	~10 min
Hemodilution (HD)-chip	Citrate	440-460	30 minutes – 3 hours after blood collection	Type I collagen and tissue thromboplastin	0.3 mm x 50 µm	1	1200	Designed for assessment of whole-blood hemostasis (primary and secondary hemostasis) in samples with low platelet count	~30 min

Table 1: Characteristics of the AR-, PL- and HD-chips used in T-TAS. AR; atheroma, Nr; number, Pl; platelet.
* 3.8% sodium citrate solution, ** the final concentration of Hirudin is 25µg/m, *** Benzylsulfonyl-D-Arg-Pro-4-amidinobenzylamide, ~; about. The flow rate in T-TAS 01 is fixed and cannot be modified by the user's manually. In T-TAS Plus, users can select flow rate from several options.

Identification of relevant studies

Eligible studies for this this scoping review were original articles investigating the usefulness of T-TAS applied to patients with bleeding disorders, thrombotic disorders or patients using antiplatelet and/or anticoagulant therapy. In order to be included in this review, studies had to meet the following criteria: (1) T- TAS experiments were performed on human samples, (2) study population was n≥10 and (3) T-TAS was used to investigate bleeding and thrombosis disorders or monitoring of anticoagulant/antiplatelet therapy. Papers that examined T-TAS in animal models or the effects of medicinal herbs on coagulation were excluded. All titles and abstracts were independently screened by two reviewers (A.M. and H.M.) and the pre-specified in- and exclusion criteria were applied during the process. The full text of potentially useful articles was then obtained. Disagreements on inclusion of articles were resolved by consensus.

Quality assessment

Two reviewers (H. M. & A.M.) independently assessed the methodological quality of the included studies. The Joanna Briggs Institute (JBI) Appraisal Tool (checklists) were used (Available from https://jbi.global/critical-appraisal-tools. Accessed on January 15, 2023). The checklist for case control studies, cohort studies, and diagnostic test accuracy studies was used, dependent on the study design. Each selected study was assigned a score from 0 to 10. The risk of bias was determined by summing the allocated scores. The final score of 7-10 was considered as low risk of bias, 4-6 was as moderate risk of bias, and 0-3 as high risk of bias. Each conflict was resolved in a joint meeting. The quality assessment checklists can be found in supplementary Table S1.

Data extraction

Data was extracted from each included article by both reviewers (A.M. and H.M.) using a pre-specified data extraction form (supplementary Table S2). The following information was extracted from each included study: first author and year of publication, title of the full text article, study location, study design, source of study population, sample size, targeted condition, used T-TAS chips and shear rates, reference standard used (golden standard laboratory test), analysis used for this review, outcome measure and authors conclusion.

RESULTS

Study selection

The literature search in PubMed, Scopus and Embase resulted in 387 results. After deduplication, 226 articles remained. During the screening of titles and abstracts, 178 articles were excluded. From the remaining 48 potentially relevant studies, the full text

version was obtained. Eventually, 28 studies were included in this scoping review (Figure 2).

Study characteristics

The study characteristics of the 28 included studies are summarized in supplementary tables S3- S6. Six studies investigated the capability of T-TAS in diagnosing congenital bleeding disorders and monitoring their treatment (supplementary Table S3). 11 articles focused on the usefulness of T-TAS in monitoring patients with anticoagulant and/or antiplatelet therapy (supplementary Table S4). Five studies assessed the ability of T-TAS in predicting the bleeding risk in different study populations (supplemenatary Table S5). Eventually, two studies used T-TAS to assess thrombogenicity in different disease entities (supplementary Table S6). One article investigated the effect of thrombocytopenia on T-TAS parameters and three studies applied T-TAS before and after hemodialysis (supplementary Table S6).

Methodological quality assessment

No studies with a high risk of bias were identified. 19 studies were determined to have a low risk of bias, the remaining 9 studies were determined to have a moderate risk of bias. No studies were excluded based on the quality assessment.

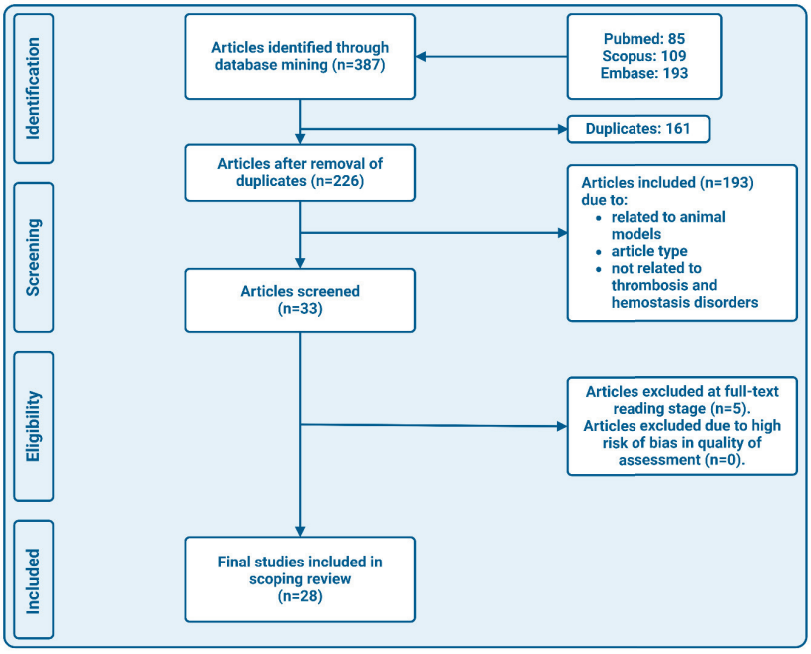

Figure 2: flowchart of the study selection.

The role of T-TAS in the diagnosis and treatment of VWD and platelet function disorders (PFD) (supplementary Table S3)

Six studies investigated the role of T-TAS in the diagnosis and treatment of patients with VWD and PFD. A short summary is given for each study and detailed study characteristics and outcomes can be found in supplementary Table S3. The study by Daidone et al. showed that T-TAS is able to detect patients with severe type 1 VWD and patients with type 2A and 2B VWD with abnormal multimers. Patients with mild type 1 VWD and type 2M VWD were not detected.

Nogami et al. investigated the usefulness of T-TAS in predicting the bleeding phenotype in type 1 VWD patients (24). There was a weak to moderate correlation between T-TAS parameters, bleeding score and VWF antigen levels.

Oggiwara et al. observed abnormal T-TAS results in one pediatric type 1 VWD patient, one adult type 2A VWD, one adult type 2N VWD patient, and one pediatric type 3 VWD (25).

A study by Ägren et al. showed reduced thrombin formation in type 3 VWD patients, which improved significantly after treatment with VWF-FVIII concentrate (26).

Lecchi et al. included patients with suspected PFD and unexplained mild to moderate bleeding (27) in whom T-TAS did not show significant differences in median parameters compared to healthy controls.

Lastly, Nakajima et al. showed that T-TAS is able to detect type 2A, 2B and 3 VWD and severe PFDs.

In conclusion, T-TAS is able to discriminate severe type 1 VWD, VWD type 2A, 2B and 3, but not mild VWD type 1 and VWD 2M. T-TAS is also able to detect severe PFD, but not milder forms of PFD. Overall, the PL-chip seems to be more sensitive for VWF-mediated platelet function than the AR-chip (Figure 3).

The role of T-TAS in monitoring anticoagulant and antiplatelet therapies (supplementary Table S4)

Eleven studies investigated the role of T-TAS in monitoring of anticoagulant and antiplatelet therapies. A short summary is given for each study and detailed study characteristics and outcomes can be found in supplementary Table S4. Idemoto et al. used T-TAS to investigate the antithrombotic effects of DOACs and showed that T-TAS is able to detect dabigatran and apixaban, but not rivaroxaban (29).

Three studies on patients with atrial fibrillation (AF) were performed using DOAC's (30-32). Ishii et al. observed significantly different T-TAS parameters in patients taking anticoagulants compared to patients not taking anticoagulants. Sugihara et al. performed T-TAS measurements at DOAC through and peak levels and showed increasingly abnormal T-TAS results at peak levels. Taune et al. evaluated thrombus formation by T-TAS and ROTEM at trough and peak levels of dabigatran (32). T-TAS parameters correlated significantly with dabigatran concentrations, as well as ROTEM clotting time (CT).

Sueta et al. showed that T-TAS is able to detect edoxaban treatment by measuring before and 7 days after the start of edoxaban treatment (33).

The study by Arima et al. performed T-TAS measurements in patients receiving (dual) antiplatelet therapy (D)APT and showed significantly different results compared to healthy controls (34). This was similar to the results measured by VerifyNow.

Zheng et al. also investigated the role of T-TAS in patients using DAPT and showed abnormal results, significantly lower than the cutoff value (35).

Lecchi et al. analyzed residual platelet function in patients treated with (D)APT using T-TAS and comparing T-TAS results with LTA results, showing a test agreement of 54% (27).

A study by Borst et al. used T-TAS and TG to investigate the effects of DAPT with or without low dose rivaroxaban in patients with acute coronary syndrome (ACS) (36) and showed that both T-TAS and TG provide significantly different results after initiation of rivaroxaban treatment.

Matsuo et al. performed T-TAS measurements in 20 pediatric Fontan patients, with and without antithrombotic therapy (aspirin and/or warfarin) and showed significantly different results compared to a control group (37).

Lastly, Kikuchi et al. used T-TAS and VerifyNow in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention (PPCI) and treated with DAPT and unfractionated heparin (UFH) (38). Results showed that T-TAS and VerifyNow levels decreased over time.

In conclusion, multiple studies observed lower AR-AUC levels in patients using DOACs, whilst PT and aPTT often showed no abnormalities in patients using DOACs (39). T-TAS could thus serve as a screening test for DOAC-use, but there was only a weak correlation between DOAC-concentration and T-TAS results. The T-TAS PL-chip is able to detect APT and DAPT and results were comparable to those of the VerifyNow (Figure 3).

The role of T-TAS in the prediction of bleeding events in patients on anticoagulant and/or antiplatelet therapy (supplementary Table S5)

Five studies investigated the role of T-TAS in predicting bleeding events in patients on anticoagulant and/or antiplatelet treatment. A short summary is given for each study and detailed study characteristics and outcomes can be found in supplementary Table S5. Ito et al. investigated the role of T-TAS in predicting periprocedural bleeding events in AF-patients treated with warfarin or DOACs and undergoing catheter ablation (CA) (40). Results showed significantly different T-TAS results in patients with bleeding complications compared to those without, whilst PT and aPTT did not differ significantly.

Nakanishi et al. performed T-TAS in patients treated with DAPT undergoing percutaneous coronary intervention (PCI) and showed significantly different T-TAS parameters in the high bleeding risk group (41).

The study by Oimatsu et al. investigated the role of T-TAS in predicting periprocedural bleeding events in coronary artery disease (CAD) patients treated with DAPT and undergoing PCI and showed significantly different results in patients with bleeding complications compared to those without (42).

Ichikawa et al. used T-TAS to investigate bleeding complications in CAD-patients using oral anticoagulation (DOAC or vitamin K antagonist) combined with DAPT (21). T-TAS results were significantly different in the bleeding complication group. Lastly, Mitsuse et al. used the T-TAS to predict the 1-year bleeding risk in CAD-patients treated with DAPT and/or DOACs and showed significantly different T-TAS parameters in bleeding patients compared to non-bleeding patients (43).

In conclusion, the T-TAS AR-chip was able to predict bleeding in CAD-patients on oral anticoagulation and in AF-patients undergoing CA (Figure 3).

The role of T-TAS in other diseases and conditions (supplementary Table S6)

Six studies investigated the role of T-TAS in other diseases and conditions. A short summary is given for each study and detailed study characteristics and outcomes can be found in supplementary Table S6. Atari et al. performed T-TAS measurements in thrombocytopenic ICU-patients, before and after platelet transfusion and showed that the HD-chip is able to detect recovery of the hemostatic function after platelet transfusion in these patients (44).

In the study by Mitic et al. T-TAS was used in end-stage renal disease (ESRD) patients, before and after hemodialysis (45). Impaired T-TAS results were seen in these patients compared to healthy controls, both before and after hemodialysis.

The study by Nakanishi et al. investigated the influence of hemodialysis on thrombogenicity in patients undergoing PCI and using DAPT using T-TAS and showed impaired T-TAS parameters in patients undergoing hemodialysis compared to those not on hemodialysis (46). Moreover, T-TAS was able to distinguish between bleeding and non-bleeding patients.

Ogawa et al. looked at haemodilution induced changes in coagulation before and after cardiopulmonary bypass (CPB) (47). Results showed prolonged T-TAS parameters after CPB compared to baseline.

Ghirardello et al. investigated platelet thrombus formation in COVID-19 patients (48) and showed significantly different T-TAS results compared to a control group, indicating impaired thrombus formation in COVID-19.

Lastly, one study by Kedzia et al. performed T-TAS measurements in patients with endometriosis and showed a prothrombotic state in these patients compared to healthy controls (49).

In conclusion, the HD-chip seems to be able to discriminate between thrombocytopenic patients with and without a bleeding tendency. Mixed results were seen in hemodialysis patients. In COVID-19, T-TAS PL-chip showed lower thrombogenicity and in endometriosis, T-TAS AR-chip showed higher thrombogenicity (Figure 3).



Figure 3: Overview of the ability of the T-TAS to detect abnormalities in different clinical situations.

DISCUSSION

This review aimed to provide a comprehensive overview of the utility of the T-TAS system in the field of thrombosis and hemostasis.

Our review shows that in VWD and PFD-patients, T-TAS seems to be able to only detect patients with severe primary hemostasis defects, and not milder forms of VWD and/or PFD. The PL-chip is able to discriminate VWD type 2A, 2B and 3, but not VWD type 1 and VWD type 2 M (23, 25). Similar results were seen in studies evaluating the PFA in VWD patients, showing that the PFA is mainly able to detect severe VWD types and abnormal VWF multimer patterns (50, 51).

The results regarding the detection of PFD are similar to results from studies showing that the PFA and multiplate are able to discriminate severe PFD's, but not mild PFD's (17). This indicates that for the diagnosis of PFD, LTA remains the gold standard.

T-TAS could be of added value in monitoring patients receiving VWF-FVIII treatment as one study suggests that the AR-chip was able to assess the differences in coagulation-dependent thrombus formation in VWD type 3 patients, before and after administration of VWF-FVIII treatment. This is not possible with the PFA, as a previous study showed that PFA normalizes after DDAVP treatment, but not always after VWF-FVIII treatment (52).

Several included studies investigated different types of anticoagulant and antiplatelet therapies. The overall conclusion is that T-TAS is able to detect the presence of warfarin, DOAC's, APT and DAPT. Several studies observed lower AR-AUC levels in patients using DOAC's, whilst PT and aPTT often showed no abnormalities. This is in concordance with previous research, showing that the detection of dabigatran and rivaroxaban is difficult with aPTT and PT, but is possible, dependent on the reagents that are used (39). The correlation between DOAC concentrations and T-TAS results was investigated in only one study, showing weak but significant correlations between DOAC concentrations and T-TAS results (32).

Studies on APT and DAPT showed that this can be detected by the T-TAS PL-chip. Comparable results were seen between T-TAS and VerifyNow in discriminating patients using DAPT (34, 38). One possible advantage of the T-TAS is its overall assessment of platelet function, whilst the VerifyNow system is designed to detect certain platelet activation routes in particular (53).

Several studies looked at bleeding risk prediction in different populations. In CAD-patients on oral anticoagulation, T-TAS was able to predict subsequent bleeding events and in AF-patients undergoing CA, T-TAS was able to predict periprocedural bleeding events. These studies were performed in rather large study populations. In general, the AR-chip seems to be a better predictor of bleeding than the PL-chip.

An important advantage of T-TAS is its ability to assess platelet function in thrombocytopenic patients, using the HD-chip. This could be relevant for patients with chemotherapy induced thrombocytopenia amongst others. Up until today, these patients receive platelet transfusions based on platelet count, and not platelet function (54). Moreover, some of these patients bleed despite platelet transfusions whilst others don't bleed at all. It would therefore be relevant to have other, more accurate parameters to base transfusion decisions on (55). One study showed that T-TAS was able to discriminate between thrombocytopenic patients with a bleeding tendency and those without and to detect recovery of hemostasis after platelet transfusion (56).

In COVID-19 patients, reduced platelet thrombogenicity measured by T-TAS was seen compared to controls in one study (48). These results are in contrast to other studies that

show increased platelet activity and reactivity in COVID-19 patients (57). In patients with endometriosis, T-TAS results showed a prothrombotic state (49). This is in accordance with one other study describing increased TG-parameters (58).

Based on the results from the included studies in this review, the T-TAS system seems to be a promising tool in different clinical situations. However, there are still some (major) limitations and knowledge gaps. First of all, there is a wide variety of T-TAS outcome parameters, leading to difficulties with interpretation, but also generalizability of the results as it is challenging to compare results from different studies when different outcome measures were used. There is now also still a lack of well-defined reference values. In supplementary Table S7, a table with reference values from all the included studies can be found. This problem could be solved in future research by either providing all the possible T-TAS parameters or by agreeing on a set of important parameters. Future research should also investigate the clinical value of each of these parameters.

Second, the majority of the included studies in this review used rather small study populations. Hence, most of the experiments need further investigation in studies with a larger sample size. Moreover, heterogeneous patient populations were investigated, especially in the studies investigating the use of anticoagulants and/or DAPT and the studies on hemodilution. This leads to difficulty in comparing these studies with each other. Therefore, it is hard to draw generalizable conclusions. In the studies investigating the use of DOAC's, the correlation of the T-TAS results with plasma concentrations of medication should be further investigated, as this is major limitation of the current studies.

An important limitation of the currently available literature on T-TAS is the lack of comparison of T-TAS with the current golden standard for platelet function testing, the LTA. Only a few studies included in this review compared T-TAS results with LTA results, but the majority of the studies didn't. Therefore, it is difficult to draw any conclusions regarding the added value of T-TAS on top of the currently available diagnostic tests. Future research on T-TAS should also include LTA results to further investigate this and whether T-TAS could increase the diagnostic yield, for example in patients with bleeding disorder of unknown cause.

Another limitation of T- TAS is the lack of integration of the endothelial function. Endothelial cells regulate platelets, coagulation, and fibrinolysis function in vivo. Although some efforts have been made to recruit vascular cells and incorporate them in the hemostasis assay, they have not been successful to date. Another challenge in this regard is that the phenotype of endothelial cells varies in different organs (59).

The clinical applicability of T-TAS is also still limited, as it is now mainly used in research settings. There is only a limited number of studies on T-TAS in the field of thrombosis and hemostasis, which is due to the fact that it is a newly manufactured product. Interpretation of the tests results will require attention as clinicians will have to be educated. Based on the currently available studies, no strong conclusions concerning the clinical added value of the T-TAS can be drawn yet.

CONCLUSION

Despite these limitations, the T-TAS method is an interesting technology that mimics the complex biological coagulation process using shear force. More research is needed, but it could eventually serve as a screening test for platelet function and coagulation. Moreover, it could be used to detect the presence of anticoagulant and/or antiplatelet medication.

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SUPPLEMENTARY MATERIALS

Table S1: Overview of the quality assessment forms.

Study	Quality assessment tool	Record number
Agren at al. 2017	JBI critical appraisal checklist for diagnostic test accuracy studies	7
Daidone et al. 2016	JBI critical appraisal checklist for diagnostic test accuracy studies	7
Lecchi et al. 2023	JBI critical appraisal checklist for diagnostic test accuracy studies	8
Nakajima et al. 2021	JBI critical appraisal checklist for diagnostic test accuracy studies	6
Nogami et al. 2016	JBI critical appraisal checklist for diagnostic test accuracy studies	6
Ogiwara et al. 2015	JBI critical appraisal checklist for diagnostic test accuracy studies	5
Ichikawa et al. 2019	JBI critical appraisal checklist for cohort studies	8
Ito et al.2016	JBI critical appraisal checklist for cohort studies	7
Mitsuse et al. 2020	JBI critical appraisal checklist for cohort studies	7
Nakanishi et al. 2021 (PCI)	JBI critical appraisal checklist for cohort studies	8
Oimatsu et al. 2017	JBI critical appraisal checklist for cohort studies	6
Atari et al. 2020	JBI critical appraisal checklist for cohort studies	6
Mitic et al.2022	JBI critical appraisal checklist for cohort studies	5
Nakanishi et al. 2021 (HD)	JBI critical appraisal checklist for cohort studies	8
Ogawa et al. 2013	JBI critical appraisal checklist for case control studies	5
Arima et al. 2016	JBI critical appraisal checklist for diagnostic test accuracy studies	8
Zheng et al. 2022	JBI critical appraisal checklist for diagnostic test accuracy studies	4 (no reference test was used)
Borst et al. 2017	JBI critical appraisal checklist for cohort studies	8
Idemoto et al. 2017	JBI critical appraisal checklist for diagnostic test accuracy studies	6

Ishii et al. 2017	JBI critical appraisal checklist for diagnostic test accuracy studies	8
Matsuo et al. 2022	JBI critical appraisal checklist for case control studies	8
Sueta et al. 2015	JBI critical appraisal checklist for diagnostic test accuracy studies	7
Sugihara et al. 2016	JBI critical appraisal checklist for diagnostic test accuracy studies	8
Taune et al. 2017	JBI critical appraisal checklist for diagnostic test accuracy studies	7
Kikuchi et al. 2020	JBI critical appraisal checklist for diagnostic test accuracy studies	7
Ghirardello et al, 2021	JBI critical appraisal checklist for cohort studies	8
Kedzia et al. 2023	JBI critical appraisal checklist for case control studies	8

Table S2: Data extraction form.

First author, year	
Title	
Study location	
Study design	
Source of study population	
Sample size	
Targeted condition	
Used chip and shear rate	
Reference standard	
Analysis used for review	
Outcome measure(s)	
Authors conclusion	

Table S3: Characteristic and finding of studies that have investigated T-TAS efficacy in congenital bleeding disorders.

First author, year	Title	Study location	Study design	Source of study population	Sample size	Targeted condition	Used chip and shear rate	Reference standard	Analysis used for review	Outcome measure(s)	Authors conclusion
Daidone et al, 2016	Usefulness of the Total Thrombus-Formation Analysis System (T-TAS) in the diagnosis and characterization of von Willebrand disease	Italy	Case control	(1)VWD patients (2)healthy controls	(1)30 (2)20	The identification and characterization of VWD-patients	PL-chip at 1167 s^{-1} AR-chip at 300 s^{-1}	Conventional coagulation assays, VWF assays	OST, OT and AUC on PL- and AR-chip in patients with VWD type 1, 2A, 2B and Vicenza	OST OT AUC	T-TAS seems to be sensitive to plasma VWF concentration and the presence of large multimers.
Nogami et al, 2016	Assessing the clinical severity of type 1 von Willebrand disease patients with a microchip flow-chamber system	Japan	Case control	(1) VWD type 1 patients (2)healthy controls	(1)50 (2)30	Evaluation of the relationship between T-TAS, bleeding score (BS) and laboratory test results in VWD type 1 in order to evaluate and predict the BS in VWD type 1 by use of T-TAS	PL-chip at 1000 and 2000 s^{-1} AR-chip at 240 s^{-1}	Conventional coagulation assays, VWF assays	Correlation of T-TAS parameters with BS and VWF laboratory test results in VWD type 1 patients	PL-T_{10} AR-T_{10}	PL-T_{10} correlated with BS and VWF:RCo levels and the Pl-chip was more sensitive than the AR-chip for VWF-mediated platelet function.

Ogiwara et al, 2015	Comprehensive evaluation of haemostatic function in von Willebrand disease patients using a microchip-based flow chamber system	Japan	Case control	(1)VWD patients (2)healthy controls	(1)5 (2)20	(a)Evaluation of thrombus formation in VWD patients before and after various treatments (b)association between thrombogenicity and bleeding score (BS)	PL-chip at 1000 s^{-1} AR-chip at 240 s^{-1}	Conventional coagulation, VWF assays, ROTEM	Evaluation of thrombus formation with T-TAS in in vitro and in vivo	PL-T_{10} PL-T_{30} PL-AUC_{10} AR-T_{10} AR-OT AR-AUC_{30}	Platelet thrombus formation in the PL-chip is highly sensitive for VWF-mediated platelet function. Fibrin-rich platelet thrombus formation in the AR-chip is relatively insensitive for quantitative and qualitative VWF defects. Combined PL- and AR-chips may be useful for the diagnosis of VWD.

Ägren et al, 2017	Monitoring of coagulation factor therapy in patients with von Willebrand disease type 3 using a microchip flow chamber system	Sweden	Case control	(1)VWD type 3 patients (2)healthy controls	(1) 10 (2) 10	Monitoring VWF-FVIII concentrate in VWD type 3	PL-chip at 1000 s^{-1} AR-chip at 240 s^{-1}	Conventional coagulation assays, VWF assays	T-TAS measurements before and after VWF-FVIII treatment in VWD type 3 patients vs. healthy controls	T_{10}^{AR} OT_{80}^{AR} AUC_{30}^{AR} AUC_{10}^{PL}	Coagulation dependent thrombus formation (AR-chip) improved after VWF-FVIII treatment in VWD type 3 patients, effect on platelet dependent thrombus formation (PL-chip) was limited.
Lecchi et al, 2023	Flow-chamber device (T-TAS) to diagnose patients suspected of platelet function defects	Italy	Cohort	(1)patients referred to the outpatient clinic with mild to moderate bleeding tendency (2)healthy controls	(1)96 (2)50	The efficacy of T-TAS to detect congenital or acquired platelet function disorders in patients with unexplained bleeding	PL-chip at 2000 s^{-1}	Conventional coagulation assays, LTA	Efficay of T-TAS to detect PFD compared to LTA in patients with a mild-to-moderate bleeding tendency	OST OT AUC	T-TAS was able to identify patients with severe PFD (δ-SPD). Milder forms of PFD were not detected with T-TAS.

Nakajima et al, 2021	A microchip flow-chamber assay screens congenital primary hemostasis disorders	Japan	Cohort	(1)VWD patients (2)PFD patients	(1)22 (2)4	The applicability of T-TAS as a screening tool for patients with suspected congenital primary hemostasis disorders	PL-chip at 1000 s^{-1} AR-chip at 240 s^{-1}	Conventional coagulation assays, flow cytometry, VWF multimer analysis, Multiplate	Ability of T-TAS to identify congenital primary hemostasis disorders	PL-T_{10} AR-T_{10}	The PL-chip distinguished all patients from healthy controls and could be a screening tool. The AR-chip distinguished VWD type 2B and 3 patients from healthy controls. PL-chip distinguished BSS and GT patients from healthy controls.

AUC: area under curve, OT: occlusion time, OST: occlusion start time, PL; platelet, AR: atheroma, FVIII: factor VIII, VWF: von Willebrand factor, T-TAS: total thrombus formation system, LTA: light transmission aggregometry, ROTEM; Rotational thromboelastometry, δ-SPD: *Delta*-storage pool diseases, BSS: Bernard-Soulier syndrome, BS: bleeding score, VWF:RCO; von Willebrand factor ristocetin cofactor, GT: glanzmann's thrombasthenia, PFD: platelet function diseases, VWF:Ag: von Willebrand antigen, S: second, S-1: per second.

Table S4: Characteristic features of studies that investigated the monitoring of anticoagulant and antiplatelet therapies by T-TAS.

First author, year	Title	Study location	Study design	Source of study population	Sample size	Targeted condition	Used chip and shear rate	Reference standard	Analysis used for review	Outcome measure(s)	Authors conclusion
Idemoto et al, 2017	Evaluation of the antithrombotic abilities of non-vitamin K antagonist oral anticoagulants using the Total Thrombus-formation Analysis System	Japan	Cohort	Hospitalized CVD patients (1) with DOAC treatment and (2) without DOAC treatment	(1)78 (2)25	Assessment of antithrombitic abilities of various NOAC's in patients with CVD	PL-chip at 2000 s^{-1} AR-chip at 600 s^{-1}	Conventional coagulation assays	Assessment of thrombogenicity in CVD patients using different DOAC's compared to CVD patients without DOAC	PL-AUC AR-AUC	AR-AUC was significantly decreased in the DOAC-treated patient group compared to patients without DOAC treatment. Also significant decreases in PL-AUC were seen in DOAC-treated patients.
Ishii et al, 2017	Direct Oral Anticoagulants Form Thrombus Different From Warfarin in a Microchip Flow Chamber System	Japan	Cohort	AF patients who underwent RFCA and were treated with (1)warfarin (2)dabigatran (3)rivaroxaban (4)apixaban	(1)29 (2)19 (3)47 (4)25	Assessment of differences in anticoagulant patterns of warfarin and DOAC's in patients with AF who had undergone RFCA	AR-chip at 600 s^{-1}	Conventional coagulation assays	Comparison of thrombogenicity before and 1 month after start treatment with DOAC or warfarin	AR_{10}-AUC_{30}	AR_{10}-AUC_{30} could be a marker for monitoring anticoagulant effects of warfarin and DOACS, but correlation with plasma DOAC concentration was weak.

Sueta et al, 2015	A novel quantitative assessment of whole blood thrombogenicity in patients treated with a non-vitamin K oral anticoagulant	Japan	Cohort	(1)patients undergoing total knee arthroplasty (TKA) and treated with edoxaban	(1)20	Evalution of thrombogenicity in patients undergoing TKA before and after start edoxaban	PL-chip at 2000 s^{-1} AR-chip at 600 s^{-1}	Conventional coagulation assays	Effect of edoxaban on thrombogenecity measured with T-TAS	PL_{18}-AUC_{10} AR_{10}-AUC_{30}	AR_{10}-AUC_{30} levels were significantly decreased 6 days after treatment with edoxaban, while PL_{18}-AUC_{10} levels were almost identical before and after edoxaban treatment.
Sugihara et al, 2016	Evaluation of the Antithrombotic Effects of Rivaroxaban and Apixaban Using the Total Thrombus-Formation Analysis System®: In Vitro and Ex Vivo Studies	Japan	Case control	(1)hospitalized AF-patients treated with rivaroxaban or apixaban (2)healthy controls	(1)16 (2)20	Evalution of the usefullness of T-TAS in monitoring the anticoagulant effects of NOAC's	PL-chip AR-chip	Conventional coagulation assays	Effect of DOAC's on thrombogenicity measured by T-TAS	PL-AUC AR-AUC	AR-AUC levels were significantly decreased in patients treated with rivaroxaban or apixaban. Anticoagulant effects of rivaroxaban and apixaban could be monitored with T-TAS.

Taune et al, 2017	Whole blood coagulation assays ROTEM and T-TAS to monitor dabigatran treatment	Sweden	Cohort	(1)AF-patients treated with dabigatran	(1)30	Evaluation of thrombus formation in AF-patients treated with dabigatran using T-TAS and ROTEM and correlation with dabigatran concentrations	AR –chip at 600 s^{-1}	Conventional coagulation assays, ROTEM	Correlation of T-TAS values to dabigatran concentrations in AF-patients compared to ROTEM	T_{10} OT AUC	T-TAS is able to detect differences in hemostasis during the dosing interval of dabigatran, but correlations with dabigatran plasma concentrations are weaker compared to ROTEM EXTEM and FIBTEM CT values.
Arima et al, 2016	Assessment of platelet-derived thrombogenicity with the total thrombus formation analysis system in coronary artery disease patients recieving antiplatelet therapy	Japan	Cohort	Patients submitted to the cardiovascular department (1)using no antiplatelet therapy (2)using aspirin only (3)using aspirin and clopidogrel	(1)56 (2)69 (3)149	The assessment of effects of different antiplatelet therapies	PL-chip at 1500 s^{-1} and 2000 s^{-1} AR-chip at 600 s^{-1}	VerifyNow P2Y12 assay	Assessment of thrombogenicity in patients using different kinds of antiplatelet therapies compared to controls	PL_{24}-AUC_{10} AR_{10}-AUC_{30}	PL_{24}-AUC_{10} accurately assessed the therapeutic effect of antiplatelet therapy.

Zheng et al, 2022	The Total Thrombus Formation (T-TAS) platelet (PL) assay, a novel test that evaluates whole blood platelet thrombus formation under physiological conditions	The Netherlands, Hungary and Sweden	Cohort	CAD-patients undergoing PCI using DAPT (1)clopidogrel + aspirin (2)prasurgel + aspirin (3)ticagrelor + aspirin	(1)22 (2)15 (3)20	Evaluation of platelet function in CAD-patients using DAPT	PL-chip at 1500 s^{-1}	NR	The ability of T-TAS to discriminate DAPT-treated patients from untreated patients	PL AUC_{10}	Compared to 260 healthy volunteers, all DAPT-treated patients had lower AUC_{10} values, suggesting excellent discrimination of DAPT-treated patients from untreated patients.
Lecchi et al, 2023	Flow-chamber device (T-TAS) to diagnose patients suspected of platelet function defects	Italy	Cohort	(1)CAD patients treated with antiplatelet therapy (2)healthy controls	(1)26 (2)50	Evaluation of the ability of T-TAS to monitor the effects of antiplatelet therapy in CAD-patients	PL-chip at 2000 s^{-1}	Conventional coagulation assays, LTA	The ability of T-TAS to assess residual platelet activity in patients on antiplatelet therapy compared to LTA	OST OT AUC	Test agreement on LTA vs. T-TAS concerning antiplatelet therapy responders was 54% (K CHOEN 0.150).
Borst et al, 2017	Inhibitory mechanisms of very low–dose rivaroxaban in non–ST-elevation myocardial infarction	Germany	Cohort	(1) NSTEMI patients treated with dual antiplatelet therapy (DAPT) and in vitro spiked with very low dose rivaroxaban	(1)40	Coagulation- and platelet-dependent thrombus formation in patients treated with DAPT and the effect of in vitro addition of rivaroxaban	PL-chip at 1500 s^{-1} AR-chip at 600 s^{-1}	Thrombin generation, LTA	OST, OT and AUC on PL- and AR-chip in DAPT-treated patients and in vitro effect of additional spiking with rivaroxaban	OST OT AUC	Additional spiking of rivaroxaban resulted in significantly reduced thrombus formation in the AR-chip. Thrombus formation on the PL-chip was not affected.

Matsuo et al, 2022	Utility of the Total Thrombus-Formation Analysis System as a Tool for Evaluating Thrombogenicity and Monitoring Antithrombotic Therapy in Pediatric Fontan Patients	Japan	Case control	(1)Fontan patients undergoing cardiac catheterization (2)healthy controls	(1)20 (2)30	Assessment of thrombogenicity using T-TAS in pediatric fontan patients treated with warfarin and aspirin	PL-chip at 1500 s^{-1} AR-chip at 600 s^{-1}	Conventional coagulation assays	Ability of T-TAS to monitor effect of aspirin and warfarin	PL_{18}-AUC_{10} AR_{10}-AUC_{30}	PL_{18}-AUC_{10} and AR_{10}-AUC_{30} could be useful to monitor the effects of aspirin and warfarin.
Kikuchi et al, 2020	Platelet-derived thrombogenicity measured by total thrombus-formation analysis system in patients with ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention	Japan	Cohort	(1)STEMI patients undergoing PPCI and treated with standard loading dose prasurgel or clopidogrel, aspirin and UFH	(1)127	Investigation of the time course and value of thrombogenecity measured by T-TAS in STEMI-patients undergoing PPCI compared with VerifyNow	PL-chip at 18 µL/min AR-chip at 10 µL/min	VerifyNow	Comparison of T-TAS and VerifyNow values in STEMI-patients undergoing PPCI	$\overline{PL_{18}}$-$\overline{AUC_{10}}$ $\overline{AR_{10}}$-$\overline{AUC_{30}}$	PRU levels increased during PPCI from baseline, whereas $\overline{PL_{18}}$-$\overline{AUC_{10}}$ levels were lower during PPCI compared with baseline. High $\overline{PL_{18}}$-$\overline{AUC_{10}}$ levels during PPCI are associated with large enzymatic infarct size. $\overline{PL_{18}}$-$\overline{AUC_{10}}$ can be a suitable marker of platelet derived thrombogenecity during PPCI in STEMI-patients.

AUC: area under curve, OT: occlusion time, OST: occlusion start time, NR: not reported, PL; platelet, AR: atheroma, T-TAS: total thrombus formation system, ROTEM; Rotational thromboelastometry, DOAC: direct oral anticoagulants, RFCA: Radiofrequency catheter ablation, DAPT: dual antiplatelet therapy, NSTEMI: non–ST-elevation myocardial infarction, LTA: light transmission aggregometry, CT: clotting time, PPCI: primary percutaneous coronary intervention, STEMI: ST-elevation myocardial infarction, PRU: $P2Y_{12}$reaction units.

Table S5: Characteristic features of studies that investigated the role of T-TAS in bleeding risk prediction.

First author, year	Title	Study location	Study design	Source of study population	Sample size	Targeted condition	Used chip and shear rate	Reference standard	Analysis used for review	Outcome measure(s)	Authors conclusion
Ito et al, 2016	Total Thrombus-Formation Analysis System (T-TAS) Can Predict Periprocedural Bleeding Events in Patients Undergoing Catheter Ablation (CA) for Atrial Fibrillation	Japan	Cohort	AF patients undergoing CA treated with (1) warfarin and (2) DOACS	(1)30 (2)98	Monitoring of the anticoagulant effects of DOACs in AF patients undergoing CA	PL-chip at 24 µL/min AR-chip at 10 µL/min	Conventional coagulation assays	Assessment of thrombogenicity in AF patients treated with DOAC or warfarin and the ability to predict periprocedural bleeding events	$\frac{PL_{24}\text{-}AUC_{10}}{AR_{10}\text{-}AUC_{10}}$	Treatment with anticoagulants significantly decreases $AR_{10}\text{-}AUC_{30}$ and $AR_{10}\text{-}AUC_{30}$ was a significant predictor of periprocedural bleeding events.
Nakanishi et al, 2021	Development and assessment of total thrombus-formation analysis system-based bleeding risk model in patients undergoing percutaneous coronary intervention	Japan	Cohort	(1)ARC-HBR positive PCI-patients (2)ARC-HBR negative PCI-patients	(1)182 (2)118	Usefullness of T-TAS in prediction of of 1-year bleeding risk in patients undergoing PCI	AR-chip at 600 s^{-1}	NR	Association between T-TAS parameters and 1-year spontaneous bleeding events in PCI-patients	$AR_{10}\text{-}AUC_{30}$	The combination of ARC-HBR and $AR_{10}\text{-}AUC_{30}$ was useful for predicting 1-year bleeding risk after PCI.

Oimatsu et al, 2017	Total Thrombus-formation Analysis System Predicts Periprocedural Bleeding Events in Patients With Coronary Artery Disease Undergoing Percutaneous Coronary Intervention	Japan	Cohort	CAD-patients undergoing elevtive percutaneous coronary intervention and treated with DAPT (1)with periprocedural bleeding (2)without periprocedural bleeding	(1)276 (2)37	Evalution of the association of periprocedural bleeding events with thrombogenecity	PL-chip at 2000 s^{-1} AR-chip at 600 s^{-1}	Conventional coagulation assays, Verifynow	Associaton of periprocedural bleeding and thrombogenicity	$\frac{AR_{10}\text{-}AUC_{30}}{PL_{24}\text{-}AUC_{10}}$	$\overline{PL_{24}\text{-}AUC_{10}}$ levels were significantly lower in patients with periprocedural bleeding events than in those without. PRU and $\overline{AR_{10}\text{-}AUC_{30}}$ levels were not significantly different between both groups.
Ichikawa et al, 2019	Impact of Total Antithrombotic Effect on Bleeding Complications in Patients Receiving Multiple Antithrombotic Agents	Japan	Cohort	Stable CAD patients treated with oral anticoagulation in addition to single or dual antiplatelet therapy	145	Evalution of anti-thrombotic effects and subsequent bleeding complications in patients using mulitple antithrombotic agents	PL-chip at 18μL/min AR-chip at 4 μL/min	Conventional coagulation assays	Antithrombotic effect and bleeding risk in patients treated with multiple antithrombotic agents	$\frac{PL_{18}-AUC_{10}}{AR_4-AUC_{30}}$	Patients with bleeding complications had low thrombogenicity compared to patients without bleeding complications. $\overline{AR_4-AUC_{10}}$ was an indepent predictor of subsequent bleeding events.

Mitsuse et al, 2020	Total Thrombus-Formation Analysis System can Predict 1-Year Bleeding Events in Patients with Coronary Artery Disease	Japan	Cohort	(1)CAD patients undergoing CAG, treated with DAPT	(1)561	To predict the 1-year bleeding events in CAD-patients treated with DAPT	PL-chip at 24μL/min AR-chip at 10μL/min	Conventional coagulation assays	The predictive role of T-TAS values in 1-year bleeding events in CAD-patients	AR_{10}-AUC_{30} PL_{24}-AUC_{10}	AR_{10}-AUC_{30} levels were lower in bleeding group compared to non-bleeding group, PL_{24}-AUC_{10} levels were not different between both groups.

T-TAS: total thrombus formation system, AUC: area under curve, OT: occlusion time, OST: occlusion start time, PL; platelet, AR: atheroma, CAD: Coronary artery disease, DOAC: direct oral anticoagulant, AF: Atrial Fibrillation, PCI: Percutaneous Coronary Intervention, PRU: P2Y12 reaction units, HBR: high bleeding risk, ARC: Academic Research Consortium, DAPT: Dual antiplatelet therapy, μl/l: microliter per liter, NR: not reported.

Table S6: Characteristic features of studies that investigated role of hemodialysis and thrombocytopenia on T-TAS markers and thrombogenecity in COVID-19 and endometriosis.

First author, year	Title	Study location	Study design	Source of study population	Sample size	Targeted condition	Used chip and shear rate	Reference standard	Analysis used for review	Outcome measure(s)	Authors conclusion
Atari et al, 2020	A modified microchip-based flow chamber system for evaluating thrombogenicity in patients with thrombocytopenia.	Japan	Cohort	(1)patients admitted to the ICU of Kagoshima University hospital requiring a platelet transfusion	(1)10	The assessment of thrombogenicity in thrombocytopenic patients	PL-chip at 1500 s^{-1} AR-chip at 600 s^{-1} HD-chip at 1100 s^{-1}	ROTEM and Multiplate Analyzer	(a)Assessment of the relationship between bleeding symptoms and values of T-TAS (b)Assessment of the effect of platelet transfusion on thrombogenicity measured by T-TAS	AUC HD OT HD	(a)HD chip is able to discriminate between hemostatic function (b)HD chip detects recovery of hemostatic function after platelet transfusion.

Mitic et al, 2022	Platelet thrombus formation in patients with end-stage renal disease before and after hemodialysis as measured by the total thrombus-formation analysis system.	Serbia	Cohort	(1)End stage renal disease (ESDR) patients treated with hemodialysis (HD)	(1)22	Evaluation of T-TAS to study thrombogenicity in ESDR patients before and after HD	PL-chip at 1500 s^{-1}	Conventional coagulation assays	Platelet – dependent thrombogenicity in ESDR patients before and after HD	PL-AUC	Platelet thrombogenicity was reduced and below cut-off value for platelet dysfunction in 17/22 patients. HD did not enhance thrombogenicity.
Nakanishi et al. 2021	Hemodialysis-related low thrombogenicity measured by total thrombus-formation analysis system in patients undergoing percutaneous coronary intervention.	Japan	Cohort	Patients undergoing PCI (1)Hemodilution patients (2)non-HD patients eGFR<60 ml/min/1.73 m^2 (3)non-HD patients eGFR ≥60 ml/min/1.73 m^2	(1)33 (2)124 (3)143	Investigation of the relationship between hemodialysis and thrombogenicity in patients undergoing PCI	PL-chip at 1500 s^{-1} AR-chip at 600 s^{-1}	NR	Relation of HD and thrombogenicity in patients undergoing PCI	PL_{18}-AUC_{10} AR_{10}-AUC_{30}	Hemodialysis was significantly associated with low PL_{18}-AUC_{10} and AR_{10}-AUC_{30} levels as measured by T-TAS.

Ogawa et al, 2013	Haemodilution-induced changes in coagulation and effects of haemostatic components under flow conditions.	Japan	Case control	(1)cardiac patients before/after cardiopulmonary bypass (CPB) (2)healthy volunteers with/without 40% dilution with saline	(1)15 (2)12	Investigation of the effects of hemodilution in CPB-patients	Chip coated with collagen and tissue factor at 1100 and $330\ s^{-1}$	Conventional coagulation assays, ROTEM	Effect of 40% hemodilution on T-TAS parameters in CPB-patients compared to healthy controls	Onset of thrombus formation (T_{on}) Growth of thrombus formation (T_{80})	$\overline{T_{on}}$ and $\overline{T_{80}}$ were prolonged after 40% hemodilution at both flow rates, both in CPB-patients and healthy controls.
Ghirardello et al, 2021	Assessment of Platelet Thrombus Formation under Flow Conditions in Adult Patients with COVID-19: An Observational Study	Italy	Cohort	Covid-19 patients admitted to the ICU, subintensive CU and low ICU. Three groups were differentiated (1)early admission (2)intermediate admission (3)late admission	(1)18 (2)19 (3)24	Evaluation of T-TAS parameters in different stages of COVID-19	PL-chip at 2000 s^{-1}	Conventional coagulation assays	Comparison of platelet-dependent thrombus formation in different stages of COVID-19	OST OT AUC	Platelet thrombus formation was impaired during the first week after admission compared to controls and patients at later disease stages. No difference in platelet thrombus formation was found among patients with different illness severity. Overall, reduced platelet thrombogenicity was observed compared to controls.

Kedzia et al, 2023	Endometriosis is associated with an increased whole-blood thrombogenicity detected by a novel automated microchip flow-chamber system (T-TAS®)	Poland	Case control	(1)Patients with surgically confirmed endometriosis (2)healthy controls	(1)23 (2)10	Investigate whole blood thrombus formation in patients with endometriosis	AR-chip at 240 $\overline{s^{-1}}$	Conventional coagulation assays	Comparison of thrombus formation in endometriosis patients vs. healthy controls	OST OT AUC	OST and OT were significantly shorter in patients than in controls. AUC was significantly higher in patients. These results indicate increased thrombogenicity in endometriosis patients.

HD: hemodialysis, AUC: area under curve, OT: occlusion time, OST: occlusion start time, PL; platelet, AR: atheroma, T-TAS: total thrombus formation system, s^{-1}: per second, ROTEM; Rotational thromboelastometry, eGFR: estimated glomerular filtration rate, T_{on}: onset of thrombus formation, m^2: cubic meter, ICU: intensive care unit, ml: milliliter, CPB: cardiopulmonary bypass, Min: minute, NR: not reported, TEG: thromboelastography, COVID-19: coronavirus disease-19, tPA: tissue thromboplastin activator.

Table S7. Reference ranges measured in healthy volunteers in different study groups.

		Hosokawa et al. 2011 N= 33	Daidone et al. 2016 N= 20	Idemoto et al. 2017 N=25	Minami et al. 2015 N=20	Nakajima et al. 2020 N= 20	Agren et al. 2017 N=20	Melnichnikova et al. 2022 N=33	Kedzia et al. 2021 N=10	Oda et al. 2021 N=122
AR Chip										
OST	Min		5, 46 (5,04-6,27)			12.1 (10-14.2)	7,2 (6,8-7,9)		10,65 (4,73-NR)	
OT	Min	8.8 ± 1.6	7,42 (6,54-8,30)				11,5 (10-12,2)		13,30 (9,99-15,40)	
AUC	/min		1868 (1808-1927)	1628 (1373-1748)		1221 (1027-1415)	1657 (1574-1755)		1468.65 (398.35 -NR)	
PL Chip										
OST	Min		2,03 (1,04-2,26)							
OT	Min		7,2 (6,11-7,53)		1.45-5.97	4.1 (2.8-5.4)				
AUC	/min		878 (784-972)	342 (278-381)	123.2-509.2	265 (171-359)	380 (362-419)	260.5 (217.3–301.9)		385.1 (248-465.6)

AR-chip: atheroma chip, OST: occlusion starting time, OT: occlusion time, AUC: area under the curve, PL-chip: platelet chip



CHAPTER 5

TPA-ROTEM IDENTIFIES HYPERFIBRINOLYSIS IN A SIGNIFICANT PROPORTION OF PATIENTS WITH BLEEDING DISORDER OF UNKNOWN CAUSE (BDUC)

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ABSTRACT

Background: In the majority of patients referred for evaluation of bleeding symptoms, no diagnosis can be made after extensive laboratory testing. These patients are classified as having a bleeding disorder of unknown cause (BDUC). Little is known about the role of the fibrinolytic pathway in BDUC patients.

Aim: To investigate the capacity of the tPA-ROTEM assay to detect fibrinolytic abnormalities in BDUC patients and to correlate these findings with fibrinolytic protein activity levels.

Methods: Patients with an increased bleeding tendency were included in a prospective cohort study. BDUC patients were identified based on an increased bleeding tendency and no abnormalities in extensive laboratory testing. The tPA-ROTEM assay was performed and plasma levels of PAI-1 activity and antigen, α2-antiplasmin, tPA antigen and TAFI were measured using ELISA assays. Pre-specified definitions of hyper- and hypofibrinolysis were applied.

Results: tPA-ROTEM identified a hyperfibrinolytic profile in 19/92 (21%) of BDUC patients. Multivariable regression analysis showed that lower PAI-1 antigen levels were independently associated with hyperfibrinolysis.

Conclusion: tPA-ROTEM identified a hyperfibrinolytic profile in 21% of BDUC patients. These findings suggest that antifibrinolytic medications may be useful to control bleeding or prevent bleeding in surgical interventions in a group of patients with BDUC.

INTRODUCTION

Patients with a clinically relevant increased bleeding tendency are often referred to a hemostasis specialist for diagnostic evaluation. However, in 60-70% of these referred patients, no cause for their bleeding symptoms can be found using currently available hemostasis laboratory tests [1, 2]. This group of patients is classified as patients with a bleeding disorder of unknown cause (BDUC). The bleeding phenotype in BDUC patients is almost indistinguishable from patients with known bleeding disorders such as von Willebrand disease and platelet function disorders [3], with the most common bleeding symptoms being mucocutaneous bleeding, including epistaxis, menorrhagia and hematomas. In addition, BDUC patients also bleed disproportionally after surgery, dental extractions and childbirth, and a high bleeding score is associated with a risk of spontaneous bleeding, similar as in VWD [4]. There are no clear guidelines concerning diagnosis, management and advice concerning heredity in BDUC patients and this forms a challenge for patients, family members and treating physicians [1] [5].

In patients with BDUC, various pathophysiological mechanisms for the increased bleeding tendency have been studied, using global and/or experimental hemostasis laboratory tests including thrombin generation [6-8], rotational thromboelastography [7, 8] and multiparameter microfluidic assays [9] amongst others. Several studies on fibrinolysis assays in patients with mild to moderate bleeding tendencies have been published, reporting inconclusive results [6, 8, 10-15].

Fibrinolysis is a complex process in which plasmin plays a key role. The fibrinolytic plasma activity is strictly controlled by both activators - tissue plasminogen activator (tPA) and urokinase (uPA) - and physiological inhibitors, such as plasminogen activator inhibitor-1 and 2 (PAI-1 and 2), α2-antiplasmin (A2AP) and thrombin activatable fibrinolysis inhibitor (TAFI) [16]. Although tPA, uPA, PAI-1, α2-AP and TAFI antigen and activity levels can be measured in plasma, the global assessment of fibrinolytic activity in the laboratory is rather challenging due to the lack of standardization of the tests and the complexity of the analysis of the test results [11].

The euglobulin clot lysis time (ECLT) is routinely applied as a functional fibrinolytic assay as it has been used to demonstrate augmented tPA in the circulation post-exercise [17] and in trauma [18], with a modification being used to diagnose functional PAI-1 deficiency [19, 20]. However, it has also been shown to have a low sensitivity for hyperfibrinolysis and is poorly validated. Moreover, the plasma handling in ECLT leads to pro-analytical changes by distorting the ratio of fibrinolytic inhibitors, thereby shifting the balance to a profibrinolytic state.

Thromboelastometry (ROTEM) is a viscoelastic test using citrated whole blood which is recalcified and supplemented with different types of coagulation activators. To our knowledge, no studies with rotational thromboelastometry (ROTEM) have previously assessed the fibrinolytic activity in patients with BDUC. Studies that have investigated rotational thromboelastometry (ROTEM) in BDUC have only used EXTEM, INTEM and FIBTEM assays, which are only applicable to assess the fibrinolytic profile in case of severe and ongoing hyperfibrinolysis [7, 8].

In the present paper we focused on assessing reduced clot resistance to tPA or even enhanced fibrinolysis in BDUC patients as a cause of their increased bleeding tendency by applying an in house developed and validated global hemostatic assay based on rotational thromboelastometry, the tPA-ROTEM [21]. Furthermore, EXTEM, INTEM and FIBTEM assays were also performed in this patient population.

The tPA-ROTEM assay was developed and validated in our center to gain more information on the fibrinolytic potential of a clot after addition of recombinant tPA. The assay, detailed by Kuiper et al 2016 [21], gives insights into the kinetics of clot formation and fibrinolysis, being able to detect hyper- and hypofibrinolytic profiles in septic patients or in those after cardiothoracic surgery [21]. Since the validation of this assay, researchers have been able to successfully detect hyper- and hypofibrinolysis in some patient populations [22] [23]. The study by Heubel-Moenen et al. [22] was able to detect a hyperfibrinolytic profile in almost half of all the chemotherapy induced thrombocytopenic (CIT)-patients. In a cohort of COVID-19 patients, Hulshof et al. [23] reported a severe hypofibrinolytic profile. Because of the high diagnostic yield of this test in other populations, it could also be able to identify a hyperfibrinolytic profile in BDUC patients.

The primary aim of this study was to investigate the ability of the tPA-ROTEM assay to detect fibrinolytic abnormalities in BDUC patients and to correlate these findings with fibrinolytic proteins such as PAI-1 and A2AP levels in plasma, in order to further unravel the bleeding mechanisms in BDUC.

MATERIALS AND METHODS

Study population

Data from the Predictors of Bleeding Evaluation in Adult Hematologic Patients with Bleeding Tendencies (ProBe-AHP) study (METC 14-4-036, NL9643), an observational study in which patients who were referred to a hematologist for bleeding evaluation, were used for this analysis. Data from forty healthy volunteers were used as a reference group for the tPA-ROTEM assay [21]. Healthy volunteers were recruited amongst employees and

students from the central diagnostic laboratory of our center. Half of them were female, with a mean age of 29 years old and a mean body mass index (BMI) of 22,3. All participating patients and healthy volunteers gave written informed consent according to the Helsinki declaration. Our patients were selected from the ProBe-AHP study from May 2014 to May 2024. The study was described in more detail previously [24]. Our sample included patients with BDUC defined as an abnormal ISTH-BAT score (≥6 for women and ≥4 for men), without abnormalities in standard coagulation tests. An overview of the standard coagulation tests that were used in this study can be found in table 1. Patients who could not discontinue medications, herbal preparations or supplements known to influence the hemostatic process were excluded from the study.

Table 1: Overview of standard hemostasis tests in the ProBe-AHP study.

Screening hemostasis tests	Complete blood count, MPV, PT, aPTT, TT, fibrinogen
Von Willebrand tests	vWF activity and vWF antigen
Coagulation factors	FII, FV, FVII, FVIII, FIX, FX, FXI, FXIII
Platelet function tests	PFA-200, LTA

MPV, mean platelet volume; PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time; vWF, von Willebrand Factor; PFA-200, platelet function analyzer 200; LTA, light transmission aggregometry; ATP-release, adenosin triphosphate release.

Blood collection and laboratory measurements

Detailed information on blood collection and laboratory measurements can be found in supplementary data 1.

Rotational thromboelastometry

The ROTEM® Delta Thromboelastometer (ROTEM; TEM International) was used to perform ROTEM assays. EXTEM, INTEM and FIBTEM assays were executed following the manufacturer's protocol. All ROTEM assays were performed within two hours after blood withdrawal. To assess the extrinsic and intrinsic coagulation, the EXTEM and INTEM assays were used respectively. The FIBTEM assay was used to investigate the contribution of fibrinogen to the clot formation. The clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF) and lysis after 60 minutes (Li60) were analyzed for each ROTEM assay.

tPA-ROTEM

The tPA-ROTEM assay was done according to the in 2016 validated and published method [21] on the ROTEM device (ROTEM, TEM international GmbH, Munich, Germany) within two hours after blood withdrawal. Tissue factor (TF), $CaCl_2$ and rtPA (Actilyse) were added to whole blood. Final concentrations of 35 pM TF, 10 mM $CaCl_2$ and 125 ng/mL

rtPA were obtained. The device temperature was set at 37 ℃ and maximum runtime was 120 minutes (7200 seconds). tPA-ROTEM parameters that were analyzed were: CT (clotting time in seconds), CFT (clot formation time in seconds), MCF (maximum clot firmness in millimeters), α (α-angle in degrees), LOT (lysis onset time in seconds), LT (lysis time in seconds). Lysis onset time is based on the time taken for the amplitude to decrease by 15% of maximum clot firmness. Lysis time is based on time taken for amplitude to drop to 10% of maximum clot firmness. Delta lysis speed (LS) was calculated at two different moments: Delta LS 100-85 was defined as the decline in %/min between maximum clot firmness and lysis onset time and Delta LS 85-10 was defined as the decline in %/min between lysis onset time and lysis time (figure 1).

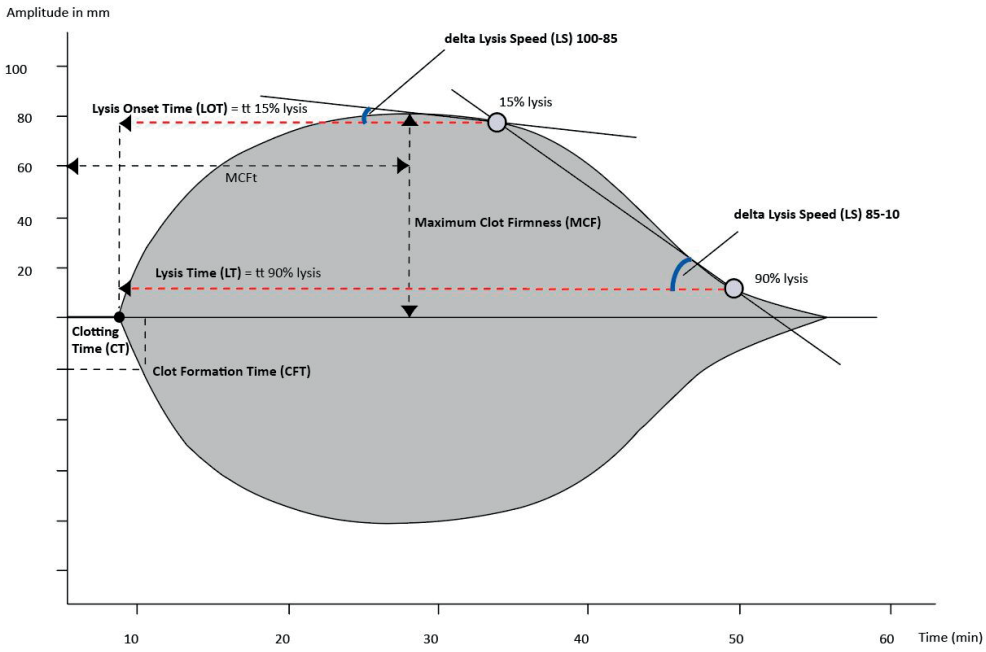

Figure 1: visual representation of tPA-ROTEM parameters, reprinted with permission from Heubel-Moenen et al [22].

Important fibrinolysis parameters are illustrated. Delta lysis speed 100-85 is defined as the decline in %/min between maximum clot firmness and lysis onset time, Delta lysis speed 85-10 is defined as the decline in %/min between lysis onset time and lysis time. Abbreviations: CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness; LOT, lysis onset time; LS, lysis speed; LT, lysis time

Definition of hyper- and hypofibrinolysis as detected by tPA-ROTEM

tPA-ROTEM parameters were validated in different patient groups and healthy controls by Kuiper et al [21]. A hyperfibrinolytic profile as measured by the tPA-ROTEM was defined by a LOT of less than 1560 seconds and/or a LT of less than 2100 seconds and/or a Delta LS 100-85 of more than 1.5%/min and/or a Delta LS 85-10 of more than 9.8%/min. A

hypofibrinolytic profile as measured by the tPA-ROTEM was defined by a LOT of more than 2940 seconds and/or a LT of more than 4620 seconds and/or a Delta LS 100-85 of less than 0.5%/min and/or a Delta LS 85-10 of less than 2.5%/min.

Statistical analysis

Continuous variables were presented as either mean ± standard deviation for normally distributed data or median ± interquartile ranges for non-parametrical data. Categorical variables were shown as counts and percentages. In case of normal distribution, differences between two groups were calculated using an unpaired t-test. In case of not normally distributed data, the Mann-Whitney U test was used and for categorical data, the Fisher's exact test was used. Statistical significance is assumed at p value <0.05. For statistical analysis, Graph Pad Prism (version 5.04) was used. In case of non-parametric data, the Kruskal-Wallis test was used and the post-hoc Dunn's Multiple Comparison Test was used. Multivariable linear regression was performed using IBM SPSS (version 28) to identify determinants of tPA-ROTEM parameters LOT, LT and delta LS, corrected for age and gender.

RESULTS

Baseline characteristics and laboratory results

A total of 92 BDUC-patients were selected from the ProBe-AHP study. Baseline characteristics and laboratory measurements are shown in Table 2. The majority of the BDUC patients was female and the mean age was 48 years old. Per definition, BDUC patients had an elevated ISTH-BAT score. All baseline laboratory measurements means were within reference ranges.

Results of tPA-ROTEM of BDUC patients compared to healthy individuals

tPA-ROTEM parameter results were compared between 40 healthy individuals and 92 BDUC-patients and are shown in figure 2. Means were all within reference ranges. The clotting time (CT) and alpha were significantly different in BDUC patients compared to healthy volunteers (67 ± 20 vs. 51 ± 11, p<0.0001 and 75 ± 4 vs. 77 ± 4, p=0.0298).

Table 2: Baseline characteristics and laboratory measurement outcomes of BDUC patients (n=92).

Patient characteristics		**BDUC (n=92)**
		Mean ± SD, n (%)
	Normal range	
Age in years		48 ± 17
Female		82 (90)
ISTH-BAT score	0-5 in women 0-3 in men	9 ± 3
Body Mass Index (BMI)	18,5-25	26,8 ± 6,1
Baseline laboratory values	**Normal range**	**Mean ± SD**
Hemoglobin (g/dL) - male	13.2-17.7	13.5 ± 2.3
Hemoglobin (g/dL) - female	11.8-15.6	12.7 ± 1.3
Leukocytes ($x\overline{10^9}$/L)	3.5-11	5.9 ± 1.8
Platelets (/microL)	130,000-350,000	261,000 ± 67,000
APTT (s)	23-32	28 ± 2
PT (s)	9.9-12.4	10.6 ± 0.5
Thrombin time (s)	13.7-17.0	16.2 ± 1.3
Fibrinogen (g/dl)	1.7-4.0	3.2 ± 0.7
VWF antigen (%)	50-150	110 ± 35
VWF activity (%)	50-150	108 ± 39

ISTH-BAT, international society on thrombosis and hemostasis bleeding assessment tool; APTT, activated partial thromboplastin time; PT, prothrombin time; VWF, von Willebrand factor.

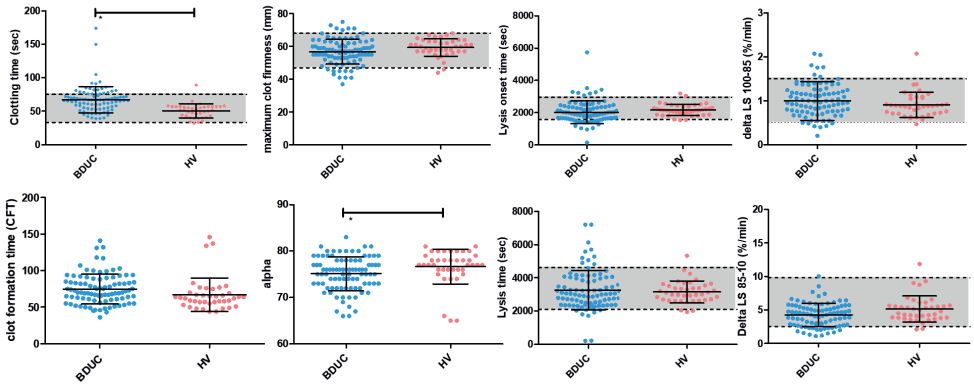

Figure 2: tPA-ROTEM results in healthy individuals (n=40) compared to BDUC patients (n=92).Results were compared between BDUC and healthy volunteers using the unpaired t-test. Statistically significant differences are indicated with * and the reference ranges are

indicated with the grey areas. CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness; LOT, lysis onset time; LT, lysis time; Delta LS 100-85, Delta lysis speed 100-85; Delta LS 85-10, Delta lysis speed 85-10

Assessment of hyperfibrinolytic profiles in BDUC patients

BDUC-patients were assessed based on LOT, LT, delta LS 85-10 and delta LS 100-85. This resulted in a total of 19/92 (21%) of BDUC-patients having a hyperfibrinolytic profile, 19/92 (21%) of BDUC-patients having a hypofibrinolytic profile and 54 BDUC-patients (58%) having a normal profile (Table 3). For further analysis, two groups were compared: one group including patients with a hyperfibrinolytic profile and one group including patients with a hypofibrinolytic or a normal profile.

Table 3: Number of BDUC patients (n=92) with hyper- or hypofibrinolysis according to tPA-ROTEM results.

Profile	BDUC n (%)	Classification criteria
Hyperfibrinolytic	19 (21)	Defined as LOT <1560 s. or LT was <2100 s. or Delta LS 85-10 was > 9.8%/min or Delta LS 100-85 was >1.5%/min
Hypofibrinolytic	19 (21)	Defined as LOT >2940 s. or LT was >4620 s. or Delta LS 85-10 was <2.5%/min or Delta LS 100-85 was <0.5%/min
Normal	54 (58)	
Total	92 (100)	

Clotting time, clot formation time and maximum clot firmness in BDUC patients with and without hyperfibrinolytic profiles

The tPA-ROTEM parameters clotting time (CT), clot formation time (CFT) and maximum clot firmness (MCF) were compared between BDUC patients with and without hyperfibrinolytic profiles and the results are shown in figure 3.There was no statistically significant difference in CT between both groups. CFT was higher in BDUC patients with as compared to patients without a hyperfibrinolytic profile, respectively (69-117) vs. 69 (56-83), p=0.01. MCF was lower in BDUC patients with a hyperfibrinolytic profile compared to patients without a hyperfibrinolytic profile, respectively 54 (45-58) vs. 58 (55-64), p=0.002.

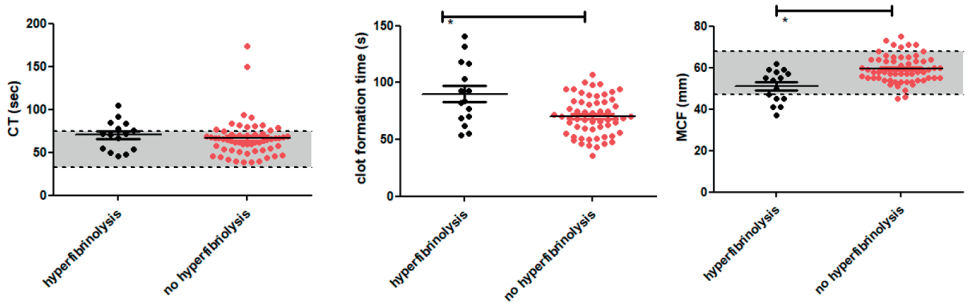

Figure 3: Comparison of clotting time, clot formation time and maximum clot firmness between BDUC patients with and without a hyperfibrinolytic profile.

Results were compared between BDUC patients with and without a hyperfibrinolytic profile using the Mann Whitney U test. Statistically significant differences are indicated with * and the reference ranges are indicated with the grey areas. CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness.

Baseline laboratory results of BDUC-patients with and without a hyperfibrinolytic profile

Baseline laboratory measurement of BDUC-patients with and without a hyperfibrinolytic profile are shown in figure 4, panel A and the fibrinolytic protein measurements are shown in figure 4, panel B. Not all parameters were available for each included patient. The percentage of missing data was 11% for PAI-1 activity, 9% for PAI-1 antigen, 26% for plasminogen, 26% for tPA-antigen and 24% for TAFI. Except for TAFI-levels, which were decreased in BDUC patients, all the means were within reference ranges (supplementary Table S1). There were no statistically significant differences in baseline laboratory measurements between both groups. Antigen levels of tPA were significantly lower in the hyperfibrinolytic group compared to the non-hyperfibrinolytic group, respectively 2,8 (1,9-3,5) vs. 4,3 (2,7-6,1), p=0,03.

Multivariable regression analysis

Multivariable regression analysis was used to identify determinants of tPA-ROTEM parameters LOT, LT, Delta LS 85-10 and Delta LS 100-85, corrected for gender and age. Table 4 shows results from a multivariable regression model in which each independent variable was added separately, corrected for age and gender. Table 4 also shows results from a multivariable regression model in which all the independent variables were added simultaneously, corrected for age and gender. A positive beta indicated an increase in SD of LOT, LT, Delta LS 85-10 or Delta 100-85 if the variable increases with one SD. A negative beta indicated a decrease in SD of LOT, LT, Delta LS 85-10 or Delta 100-85 if the variable increases with one SD. Table 4 shows that TAFI and PAI-1 antigen and activity have a statistically significant effect on tPA-ROTEM parameters. However, when using

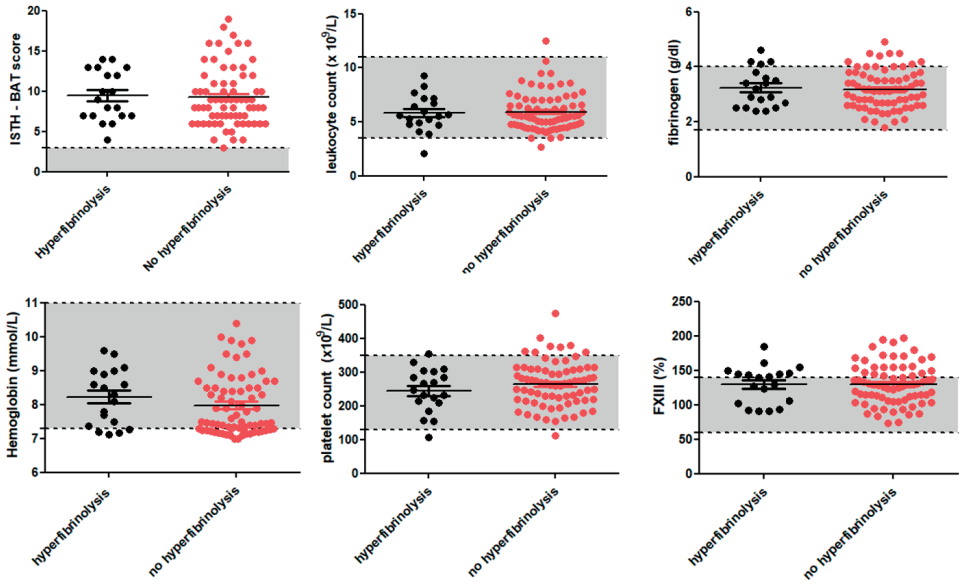

Figure 4, panel A: Baseline laboratory measurements and ISTH-BAT scores in BDUC patients (n=92).

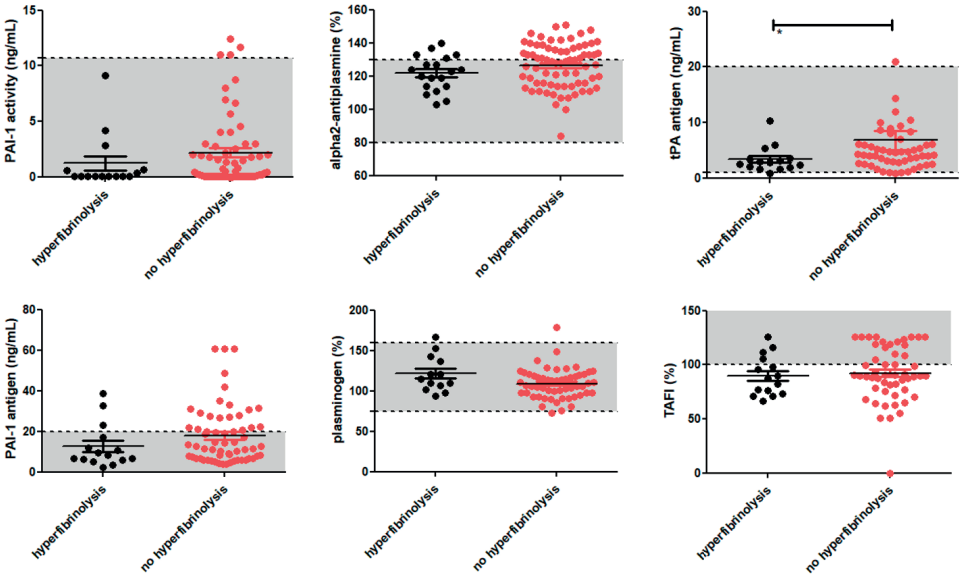

Figure 4, panel B: Fibrinolysis protein measurements in BDUC patients (n=92).

Results were compared between BDUC patients with and without hyperfibrinolytic profiles using the Mann Whitney U test. Statistically significant differences are indicated with * and the reference ranges are indicated with the grey areas. α2-AP, alpha2-antiplasmin; ISTH-BAT, international society on thrombosis and hemostasis bleeding assessment tool; PAI-1, plasminogen activator inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor; tPA, tissue plasminogen activator.

one multivariable linear regression model with all independent variables included simultaneously, only PAI-1 antigen has a statistically significant effect on tPA-ROTEM parameters, indicating that lower levels of PAI-1 antigen were independently associated with hyperfibrinolysis in BDUC-patients (table 4).

Table 4: Determinants of tPA-ROTEM in BDUC-patients (n=92) using a separate multivariable linear regression models and one multivariable regression model.

Separate multivariable regression model								
	Lysis onset time		**Lysis time**		**Delta LS 85-10**		**Delta LS 100-85**	
	Beta	***p***	***Beta***	***p***	***Beta***	***p***	***Beta***	***p***
Prothrombin	0.098	0.4	0.176	0.1	-0.097	0.4	-0.126	0.2
FXIII	-0.029	0.8	0.058	0.6	0.042	0.7	-0.063	0.6
TAFI	0.311	**0.017**	0.263	**0.044**	-0.075	0.6	-0.215	0.1
PAI-1 activity	0.360	**0.002**	0.381	**0.001**	-0.164	0.2	-0.164	0.2
PAI-1 antigen	0.441	**<0.001**	0.465	**<0.001**	-0.158	0.2	-0.215	0.1
tPA antigen	0.138	0.3	0.142	0.3	0.044	0.3	-0.040	0.8
α2-antiplasmin	0.079	0.5	0.154	0.2	-0.001	0.9	-0.151	0.2
Plasminogen	-0.208	0.1	-0.038	0.8	-0.109	0.4	0.064	0.6
Fibrinogen	0.034	0.8	-0.077	0.5	0.040	0.7	0.000	0.9
One multivariable regression model								
	Lysis onset time		**Lysis time**		**Delta LS 85-10**		**Delta LS 100-85**	
	Beta	***p***	***Beta***	***p***	***Beta***	***p***	***Beta***	***p***
Prothrombin	0.046	0.8	0.096	0.6	-0.409	0.1	-0.149	0.4
FXIII	-0.119	0.5	-0.207	0.2	0.274	0.2	0.256	0.2
TAFI	0.283	0.1	0.071	0.6	0.087	0.6	-0.115	0.5
PAI-1 activity	-0.338	0.2	-0.140	0.6	-0.177	0.6	0.283	0.3
PAI-1 antigen	0.958	**0.002**	0.669	**0.04**	0.052	0.9	-0.793	**0.02**
tPA antigen	-0.199	0.3	-0.044	0.8	0.055	0.8	0.070	0.7
α2-antiplasmin	0.149	0.3	0.152	0.4	-0.008	0.9	-0.051	0.8
Plasminogen	-0.188	0.3	-0.020	0.9	-0.115	0.6	0.051	0.8
Fibrinogen	0.194	0.2	0.028	0.9	0.050	0.8	-0.113	0.5

*corrected for age and gender
Statistically significant outcomes are marked in bold. PAI-1, plasminogen activator inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor; tPA, tissue plasminogen activator.

Results of ROTEM-EXTEM, -INTEM and –FIBTEM in BDUC-patients

Results of the EXTEM-, INTEM- and FIBTEM-ROTEM assays are shown in supplementary Table S2. EXTEM, INTEM and FIBTEM data was available from 87/92 of the BDUC patients. The means for all the ROTEM parameters were within reference ranges, except for the FIBTEM-Li60, which was just below the reference range.

DISCUSSION

This is the first study to investigate the use of tPA-ROTEM in BDUC patients. Apart from the tPA-ROTEM assay, fibrinolytic proteins were also assessed. Our results showed that (1) in 21% of the BDUC patients, a hyperfibrinolytic profile in the tPA ROTEM was found and (2) reduced PAI-1 antigen levels were independently associated with hyperfibrinolysis. This might indicate that the fibrinolysis parameters are within the lower functional range of their distribution, shifting the balance towards increased clot lysis leading to an increased bleeding tendency.

The results from the tPA-ROTEM analysis are in line with results from previous studies investigating fibrinolysis in BDUC patients, also showing hyperfibrinolytic patterns in (part of) their BDUC populations. In a cohort of 382 BDUC patients, clot lysis was assessed using a turbidity assay, showing a shorter clot lysis time (CLT) compared to healthy controls [6]. However, a similar turbidity assay was done in another cohort of 120 BDUC patients, in whom a prolonged CLT was seen compared to healthy controls, which is contradictory for the presence of hyperfibrinolysis [8]. A modified ECLT, measured before and after venous compression, provided a relatively high diagnostic yield in a group of 84 BDUC patients [11], in which 37 patients were diagnosed with a presumptive fibrinolytic disorder based on abnormal ECLT values and decreased PAI-1 antigen and activity levels. Shorter ECLT values were also seen in another cohort of 45 BDUC patients [12]. The use of different fibrinolysis assays and the heterogeneity of BDUC cohorts in these different studies limits the drawing of strong conclusions from these results.

Measurement of fibrinolytic proteins showed lower tPA levels in patients with a hyperfibrinolytic profile than in patients without a hyperfibrinolytic profile, which is not what would be expected. Other studies on fibrinolysis in BDUC patients have also looked at fibrinolysis proteins. In a cohort of 270 BDUC patients, tPA, PAI-1, tPA-PAI-1 complexes, TAFI, A2AP and D-dimer levels were determined. tPA activity, TAFI, A2AP and fibrinogen levels were increased and tPA-PAI-1 complex levels were decreased in BDUC patients compared to healthy volunteers. These findings suggest increased fibrinolytic activity [10]. Another study investigated PAI-1 antigen and activity levels, α2-antiplasmin and tPA-activity levels in 84 BDUC patients [11]. In 37/84 BDUC patients, a presumptive fibrinolytic

disorders was seen based on lower PAI-1 activity and antigen levels, but primarily based on abnormalities in euglobulin clot lysis time (ECLT). Comparable results were found in a cohort of 45 patients, in whom a shorter ECLT was seen compared to controls [12]. In this cohort, higher TAFI levels were seen, whilst in case of hyperfibrinolysis, lower levels of TAFI would be expected [12]. Higher TAFI levels were also found in a cohort of 116 BDUC patients compared to a group of controls, suggesting that the role of TAFI in the bleeding pathogenesis in this patient group is rather limited [25]. In our study, the mean fibrinolysis protein levels where within reference ranges. Limitations to the measurement of fibrinolytic parameters are the poor availability in hospital laboratories and the lack of consensus on cut-off levels to define deficiencies. Moreover, several factors are known to influence fibrinolytic protein levels and should be considered when interpreting data. For instance, PAI-1 is known the have a diurnal variation, with higher PAI-1 levels in the morning. Secondly, an association was found between PAI-1 levels and BMI, with higher PAI-1 levels in case of a higher BMI. In our patient population, the mean BMI was rather high (26,8), and could possibly have an influence on the PAI-1 levels. Oral contraceptives (OAC's) were shown to be associated with PAI-1 levels as well, with lower PAI-1 levels in women on OAC's [13]. In our study population, 23% of the female patients were using OAC's and there was no statistically significant difference in PAI-1 levels between patients with and without OAC's.

In our study, the tPA-ROTEM assay was able to identify individuals with a hyperfibrinolytic profile in whom hyperfibrinolysis may contribute to the bleeding tendency. The tPA-ROTEM assay results indicate that part of the BDUC patients have reduced clot resistance to tPA, causing a hyperfibrinolytic profile. This can be interpreted as additional evidence on top of previous studies suggesting that hyperfibrinolysis can be (one of) the explanatory mechanism(s) of the bleeding mechanism in a subgroup of BDUC-patients. On the other hand, patients with a hypofibrinolytic profile were also identified within our BDUC-group. These findings confirm the hypothesis that the fibrinolysis pathway is not the sole explanatory mechanism in all BDUC-patients. In BDUC-patients with a hypofibrinolytic profile, we hypothesize the presence of different causes for their increased bleeding tendency, leading to compensatory mechanisms resulting in decreased fibrinolysis potency.

Hyperfibrinolysis has also been shown to be associated with specific clinical situations, such as acute anxiety and the use of antidepressants [26]. An increased release of tPA, in acute anxiety could cause increased fibrinolysis. On the other hand, acute anxiety is also known to cause increased levels of FVIII, VWF and fibrinogen, suggesting a prothrombotic state. In these specific cases, antifibrinolytics should be prescribed with caution. In

chronic anxiety and depression, increased PAI-1 levels have been demonstrated [27, 28]. In our study population, patients using selective serotonin reuptake inhibitors (SSRI's) were excluded because of the known effect of these drugs on the hemostatic process [29, 30].

Apart from the tPA-ROTEM assay, other functional fibrinolysis tests are available. The majority of them is only used in research settings, have poor predictive values in case of bleeding disorders (presumptive hyperfibrinolysis) [16, 31] and are therefore not included in clinical settings [32]. It is important to consider that measurement of fibrinolytic and antifibrinolytic proteins in plasma remains challenging, especially in PAI-1 decreased levels [33]. There is thus still a lack of standardized and broadly available global fibrinolysis assays.

A limitation of our study is the fact that the role of the endothelial damage and/or activation cannot be studied in the tPA-ROTEM assay. These mechanisms possibly play in important role in the pathophysiological mechanisms in these patients. Furthermore, clinical gestalt was not considered in the selection of BDUC patients from the ProBe-AHP cohort, which could have caused selection bias since the ISTH-BAT score has its own limitations [34]. Moreover, the BDUC population is highly heterogeneous.

Future research on this topic should aim for the development of standardized fibrinolysis assays in order to be able to compare results from different centres and to be able to analyse these results in larger, possibly combined BDUC cohorts. A fibrinolysis assay that could be of interest is the Lysis Timer, a commercially available plasmatic fibrinolysis assay [35]. According to currently available literature, this assay has not been studied in BDUC-patients yet. Standardization of fibrinolysis tests and consensus on which fibrinolysis assays should be used between different centres could lead to the ability of identifying BDUC patients with hyperfibrinolysis, explaining part of the underlying bleeding mechanism in this heterogeneous group. Furthermore, it would be interesting to identify families with hyperfibrinolytic profiles and possibly confirm the fibrinolytic defect on a genetic level.

Moreover, a future study investigating treatment of BDUC patients with a hyperfibrinolytic profile with tranexamic acid (TxA), an antifibrinolytic drug, compared with BDUC patients without a hyperfibrinolytic profile, could possibly lead to evidence for more targeted treatment, as currently, there are no clear targeted treatment guidelines for BDUC patients [5]. Previous studies on current practice in BDUC have shown that TxA is widely used in BDUC patients as a prophylactic treatment in case of dental or surgical interventions, based on its availability, low cost and efficacy in other bleeding disorders [36]. It is also effective when applied locally as it inhibits salivary plasminogen. Three studies investigating the

effectiveness of prophylactic treatment using TxA (and/or desmopressin) in case of dental or surgical treatment showed this treatment was effective in up to 70-90% of the BDUC patients, however an increased peri-interventional bleeding risk was still present in BDUC patients [7, 37, 38].

CONCLUSION

The tPA-ROTEM assay was able to identify a hyperfibrinolytic profile in 21% of BDUC patients, which may be amenable to treatment. The results showed that the lysis parameters LOT, LT and delta LS are in agreement with the expected changes in fibrinolytic proteins in case of hyperfibrinolysis. Fibrinolytic abnormalities can thus explain the bleeding problems in part of the BUDC-patients.

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SUPPLEMENTARY MATERIALS

Supplementary data 1: blood collection and laboratory measurements

Blood collection

Patients were asked to not consume fat-containing products 4 hours before blood withdrawal. Medications interfering with diagnostic tests had to be stopped at least 7 days prior to blood withdrawal. Blood was withdrawn from the antecubital vein by puncture using a 21G needle. EDTA blood (BD Vacutainer; Becton Dickinson) was used for complete blood counts. Citrated blood (Vacuette, Greiner bio-one) was used for tPA-ROTEM tests. The remainder of the citrated blood was centrifuged at 2490 G for five minutes at 18 ℃ (Universal 320 R, Hettich Zentrifugen) and a second time at 10000 G for ten minutes at 18℃ to obtain platelet poor plasma (PPP). PPP was used for routine coagulation tests (prothrombin time (PT), activated partial prothrombin time (aPTT), fibrinogen and thrombin time (TT). Remaining PPP was frozen at -80 ℃. Frozen PPP was used for determination of coagulation factor levels, von Willebrand factor levels, PAI-1 activity and antigen levels, TAFI levels, α2-antiplasmin levels, plasminogen levels and tPA activity levels.

Laboratory measurements

Hemoglobin (Hb), platelet count and white blood cell (WBC) levels were measured on a Sysmex XN-9000 analyzer. Routine hemostasis tests, consisting of PT (Innovin; Siemens), aPTT (ActinFSL; Siemens), Thrombine Time (Thromboclotin; Siemens), fibrinogen (Clauss Method, Thrombine reagent; Siemens) and FXIII activity (Berichrom FXIII, Siemens), were measured on a Sysmex CS-2500 coagulation analyzer. Coagulation factor levels FVIII, FII (neoplastine R), FV, FVII, FVIII, FIX, FX (neoplastine R) and FXI were measured with a clotting assay using factor depleted plasma on a Sta-R apparatus (Stago Diagnostics, Paris, France). VWF antigen and activity levels were measured on a Sysmex CS2100i analyzer (VWF antigen Reagens and VWF Activity Reagens, Siemens). Plasminogen (Stachrom Plasminogen) and α2-antiplasmin (Stachrom Antiplasmin) were measured by chromogenic assays on Sta-R (Stago diagnostics, Paris, France). Bio-immunoassays were used to measure plasminogen activator inhibitor-1 (PAI-1) activity levels, tissue plasminogen activator (tPA) and thrombin activatable fibrinolysis inhibitor (TAFI) (Zymutest, Hyphen, Biomed). PAI-1 antigen levels were measured using an ELISA test (Zymutest, Hyphen, Biomed).

Supplementary table S1: Fibrinolysis proteins in BDUC patients (n=92).

Fibrinolysis protein	Normal range	BDUC (n=92)
		Mean ± SD
PAI-1 activity (%)	<10.7	2.159± 3.535
[%missing data]		[11]
PAI-1 antigen (ng/ml)	0-20	17 ± 14.44
[%missing data]		[9]
α2-AP (%)	80-130	125 ± 13
[%missing data]		[0]
Plasminogen (%)	75-160	111 ± 19.65
[%missing data]		[26]
tPA antigen (ng/ml) [%missing data]	1-20	4.721 ± 3.481
		[26]
TAFI (%)	100-172	92.77 ± 21.25
[%missing data]		[24]
FXIII (%)	60-140	130 ± 28.94
[%missing data]		[0]

PAI-1, plasminogen activator inhibitor 1; α2-AP, alpha2-antiplasmin; tPA, tissue plasminogen activator; TAFI, thr ombin activatable fibrinolysis inhibitor; ECLT, euglobulin clot lysis time.

Supplementary table S2: Results of the EXTEM-, INTEM- and FIBTEM-ROTEM assays in BDUC patients.

ROTEM-EXTEM parameter	**Reference ranges**	**BDUC patients (n=87) Mean ± SD**
CT (s)	49-77	64 ± 9
CFT (s)	44-129	76 ± 18
MCF (mm)	55-74	65 ± 5
Li60 (%)	91-100	92 ± 3
ROTEM-INTEM parameter	**Reference ranges**	**BDUC patients (n=92) Mean ± SD**
CT (s)	151-209	179 ± 22
CFT (s)	45-121	64±13
MCF (mm)	52-71	64 ± 5
Li60 (%)	89-100	91 ± 3
ROTEM-FIBTEM parameter	**Reference ranges**	**BDUC patients (n=92) Mean ± SD**
CT (s)	43-100	60 ± 8
CFT (s)	-	823 ± 1231
MCF (mm)	5-19	19 ± 6
Li60 (%)	100-100	97 ± 5

CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness; Li60, lysis after 60 minutes.



CHAPTER 6

DIAGNOSTIC VALUE OF THROMBIN GENERATION USING DIFFERENT TRIGGER REAGENTS IN PATIENTS WITH BLEEDING DISORDER OF UNKNOWN CAUSE (BDUC)

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ABSTRACT

Background: In 40–70% of patients referred for evaluation of an increased bleeding tendency, no diagnosis is established despite extensive laboratory workup, and these patients are classified as having a bleeding disorder of unknown cause (BDUC). The thrombin generation assay (TGA) is a global hemostasis assay that may capture subtle coagulation abnormalities in this population, but previous studies using different trigger reagents have yielded inconsistent results. This study assessed whether the TGA can identify deviations from reference values in patients with BDUC, and determined which trigger reagent (an intrinsic contact activator, PPP Reagent INT; a low tissue factor extrinsic reagent, PPP Reagent LOW; or a modified low phospholipid version, PPP Reagent LOW 1:5 PL) is most informative in this population.

Methods: TGA was performed using the calibrated automated thrombography method in 83 BDUC patients (ProBe-AHP study) and 107 healthy volunteers, with all three trigger reagents. Lag time, endogenous thrombin potential (ETP), peak height, and velocity index were compared between groups, reference ranges (2.5th–97.5th percentiles) were established from the healthy volunteers, and the discriminative ability of each parameter was assessed by receiver operating characteristic (ROC) analysis. P values for between-group comparisons and ROC analyses were adjusted separately for multiple testing using the Bonferroni-Holm method.

Results: BDUC patients showed a significantly prolonged lag time with PPP Reagent INT and PPP Reagent LOW 1:5 PL compared with healthy volunteers; with PPP Reagent LOW 1:5 PL, ETP was additionally reduced. No significant between-group differences were observed with PPP Reagent LOW. Overall, 21/83 (25.3%) BDUC patients had at least one TGA abnormality pointing towards a bleeding profile across reagents. Lag time with PPP Reagent INT showed the strongest discriminative ability (AUC 0.691), whereas PPP Reagent LOW 1:5 PL showed the broadest discriminative profile, with AUCs ranging from 0.583 to 0.634 and significant discrimination for lag time and ETP. TGA parameters correlated with aPTT and coagulation factors II, VIII, IX, and X, but not with the ISTH-BAT score.

Conclusion: TGA detects deviations from reference values, predominantly a prolonged lag time, in a subset of BDUC patients. No single trigger reagent was uniformly superior: PPP Reagent LOW 1:5 PL provided the broadest discriminative profile across all four parameters, while lag time with PPP Reagent INT showed the strongest individual discrimination. These findings support impaired thrombin generation as a reproducible feature of BDUC.

INTRODUCTION

In approximately 40–70% of patients referred to a hemostasis specialist for evaluation of an increased bleeding tendency, no diagnosis can be established despite extensive laboratory workup. These patients are classified as having a bleeding disorder of unknown cause (BDUC) (1). The bleeding phenotype of BDUC is comparable to that of patients with known mild bleeding disorders, such as von Willebrand disease and platelet function disorders (2). BDUC predominantly affects women, and the most common bleeding symptoms are mucocutaneous, including epistaxis, hematomas, and heavy menstrual bleeding, as well as peri-operative or dental bleeding (3). A diagnosis of BDUC requires an elevated International Society on Thrombosis and Hemostasis Bleeding Assessment Tool (ISTH-BAT) score in combination with normal findings on a standardized set of hemostasis tests, as defined by a recent ISTH communication (4). This diagnostic set includes a complete blood count and blood smear, prothrombin time (PT), activated partial thromboplastin time (aPTT), Clauss fibrinogen, thrombin time, von Willebrand factor (VWF) antigen and activity levels, factor (F) VIII, FIX, and FXI levels, and platelet function testing by light transmission aggregometry (LTA).

The thrombin generation assay (TGA) provides a global, functional measure of the coagulation system, quantifying thrombin formation from initiation through inactivation, thereby capturing both the procoagulant and anticoagulant contributions to hemostasis (5, 6). The TGA results in a thrombogram from which multiple parameters are derived: the lag time (time to initiation of thrombin generation in min), peak height (maximal thrombin concentration in nM), endogenous thrombin potential (ETP; area under the curve in nM·min), and velocity index (rate of thrombin generation in nM/min) (Figure 1) (6, 7). This is in contrast to the PT and aPTT, which only reflect the initiation phase of thrombin generation until a fibrin clot is formed (8). Thrombin generation can be triggered via the extrinsic pathway using tissue factor (TF) and phospholipids, or via the intrinsic pathway. he choice and concentration of the trigger reagent influence the sensitivity of the assay, particularly in populations with subtle hemostatic defects. The TGA is thus an informative tool to assess the overall coagulation profile and the balance between pro- and anticoagulant pathways, reflecting the complex interaction of coagulation proteins and enzymes rather than focusing on individual factor levels.

In BDUC patients, it is hypothesized that different bleeding mechanisms underlie the increased bleeding tendency (3). Different pathophysiological mechanisms have been studied in BDUC populations, including the fibrinolytic system, platelet function, and the overall coagulation profile using global hemostasis assays. Previous studies investigating TGA in BDUC have yielded inconsistent results. Studies using low (~1 pM) to moderate (~5 pM) TF concentrations found no significant differences in TGA parameters between BDUC

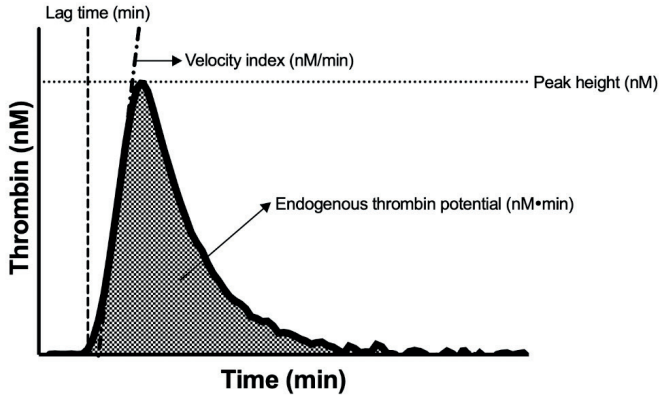


Figure 1: The thrombogram and its parameters.

patients and healthy controls (9-12). In contrast, studies using lower TF concentrations identified abnormalities in a substantial proportion of BDUC patients: Hofer et al. reported a prolonged lag time with decreased velocity index and ETP using a TF concentration <0.3 pM, MacDonald et al. reported a prolonged lag time in 26% of patients using 1.5 pM TF, and Cornette et al. identified abnormal TGA parameters in over 60% of patients using the ST Genesia and Calibrated Automated Thrombography (CAT) platforms (13-15). Most recently, Dreier et al. confirmed impaired thrombin generation in an independent cohort using the fully automated Ceveron S100 platform with two reagents of differing tissue factor concentration (very low and low) (16). Taken together, these results highlight that the diagnostic yield of TGA in BDUC is highly dependent on the trigger reagent and TF concentration used. Moreover, the potential of intrinsic pathway activation as an alternative trigger strategy has not yet been explored in this population.

The present study has two main aims. First, to assess whether the TGA can identify deviations from reference values in patients with BDUC. Second, to determine which trigger reagent is most informative in this population by comparing an intrinsic trigger reagent (PPP Reagent INT), a low tissue factor extrinsic reagent (PPP Reagent LOW), and a modified version with diluted phospholipids (PPP Reagent LOW 1:5 PL).

MATERIALS & METHODS

Study population

Patients, plasma and data from the Predictors of Bleeding Evaluation in Adult Hematologic Patients with Bleeding Tendencies (ProBe-AHP) study (METC 14-4-036, NL-omon 27868) of the Maastricht University Medical Center+ (MUMC+) were used for this study. The ProBe-AHP study has been described in detail elsewhere (17, 18). Briefly, patients were eligible

for inclusion if they were referred for evaluation of an increased bleeding tendency. BDUC was defined as an elevated International Society on Thrombosis and Hemostasis Bleeding Assessment Tool (ISTH-BAT) score (≥6 for women, ≥4 for men) in combination with normal findings on standard hemostasis testing (Supplementary table 1). A total of 83 BDUC patients were included. As a reference population, plasma samples from 107 healthy volunteers from the institutional normal pool were used. All participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the local medical ethics committee of the MUMC+.

Blood collection

In all participants, venous blood was drawn using 21-gauge Vacutainer Eclipse needles (Becton Dickinson, Plymouth, UK) and collected in Vacuette 3.2%(w/v) sodium citrate tubes (Greiner Bio-One, Kremsmunster, Austria). Platelet-poor plasma (PPP) was prepared by double centrifugation: first at 2500 × g for 5 minutes, followed by centrifugation of the supernatant at 10 000 × g for 10 minutes, both at room temperature (19). The PPP was snap-frozen using liquid nitrogen before storage at −80 °C until later use.

Baseline laboratory measurements

BDUC

Hemoglobin, platelet count, and leukocytes were measured on a Sysmex XN-9000 analyzer (Sysmex, Kobe, Japan). Routine hemostasis tests, consisting of prothrombin time (PT; Innovin; Siemens, Marburg, Germany), activated partial thromboplastin time (aPTT; Actin FSL; Siemens, Marburg, Germany), fibrinogen activity (Clauss method; Thrombin Reagent; Siemens, Marburg, Germany), and factor XIII (FXIII) activity (Berichrom FXIII; Siemens, Marburg, Germany), were performed on a CS-2500 coagulation analyzer (Sysmex, Kobe, Japan). Coagulation factor levels (FII, FV, FVII, FVIII, FIX, FX, FXI, and FXII), von Willebrand factor (VWF) antigen and VWF activity (ristocetin cofactor), protein C and antithrombin were measured according to local standard procedures.

Healthy volunteers

Hemostasis measurements were performed on a Siemens CN3000 coagulation analyzer (Siemens, Marburg, Germany) using Siemens reagents. Measurements included prothrombin time (PT; Innovin), activated partial thromboplastin time (aPTT; Actin FS), fibrinogen activity (Clauss method), VWF antigen, VWF activity, coagulation factor levels (FII, FV, FVII, FVIII, FIX, FX, FXI, FXII, and FXIII), antithrombin, protein C (chromogenic assay), and free protein S.

Thrombin generation assay

Thrombin generation was determined as previously described by Urlings et al. (2026), using the CAT method on a Fluoroskan Ascent Fluorimeter (Thermo Fisher Scientific,

Waltham, MA, USA) equipped with a 390/460 nm filter and Thrombinoscope 5.0 software (Thrombinoscope B.V., Maastricht, The Netherlands) (20). Each well contained 80 µL plasma and 20 µL trigger reagent. After incubation for 10 minutes at 37 °C, coagulation was initiated by adding 20 µL FluCa reagent containing 2.5 mM Z-Gly-Gly-Arg-AMC and 100 mM $CaCl_2$, resulting in a total reaction volume of 120 µL. Each run lasted 90 minutes. All measurements were performed in duplicate and calibrated against duplicate samples containing 20 µL of thrombin calibrator (Thrombinoscope B.V., Maastricht, The Netherlands). The following four parameters were derived from each thrombogram: lag time, endogenous thrombin potential (ETP), peak height, and velocity index.

Trigger reagents

Thrombin generation was activated using three trigger reagents. PPP Reagent INT activates the intrinsic coagulation pathway via a soluble contact activator (20). PPP Reagent LOW is an extrinsic trigger containing a low tissue factor (TF) concentration. A modified version of PPP Reagent LOW was also used in which the phospholipid (PL) concentration was diluted 1:5 with unaltered TF concentration (referred to as PPP Reagent LOW 1:5 PL). This trigger reagent was included based on the rationale that reducing the availability of negatively charged surfaces may amplify subtle coagulation defects, thereby increasing assay sensitivity in a population with mild hemostatic abnormalities. All trigger reagents were manufactured by Coagulation Profile B.V. (Maastricht, The Netherlands). Inter- and intra-assay coefficients of variation (CVs) were assessed in normal pool plasma according to CLSI Guideline EP05-A3. Measurements were performed in duplicate or triplicate, depending on plasma availability, over 26 consecutive days.

Reference ranges

Reference ranges for thrombin generation were established from the 107 healthy volunteers from the institutional normal pool, using all three trigger reagents. For each reagent and parameter, reference ranges were calculated as the 2.5th to 97.5th percentiles. A TGA result was classified as abnormal in the bleeding direction when the lag time was above the upper reference limit and/or the ETP, peak height, or velocity index was below the lower reference limit. A result was classified as abnormal in the direction of a thrombotic phenotype when the lag time was below the lower reference limit and/or the ETP, peak height, or velocity index was above the upper reference limit.

Statistical analysis

Continuous variables are presented as median (interquartile range [IQR]) for non-normally distributed data and as mean ± standard deviation (SD) for normally distributed data, as determined by the Shapiro-Wilk test. Categorical variables are shown as counts and percentages. Results reported as >150% despite additional dilution (up to 1:8 for factor XIII activity and 3:8 for free protein S in healthy volunteers) were included as right-censored

observations in the baseline summaries but excluded from the correlation analyses. To compare TGA parameters between BDUC patients and healthy volunteers, the Mann-Whitney U test or Welch's t-test was applied as appropriate. To control the familywise error rate across the 12 simultaneous comparisons (3 reagents × 4 parameters), p values were adjusted using the Bonferroni-Holm method.

Associations between TGA parameters and clinical and laboratory variables were assessed using Spearman rank correlation coefficients; p values were adjusted for multiple comparisons using the Bonferroni–Holm method. Spearman correlations were also computed independently in BDUC patients and healthy volunteers, and differences between correlation coefficients were tested using Fisher's z-transformation. The discriminative ability of TGA parameters to distinguish BDUC patients from healthy volunteers was assessed by receiver operating characteristic (ROC) analysis. Areas under the curve (AUCs) with 95% confidence intervals were calculated using the DeLong method. P values for the null hypothesis of AUC = 0.5 were obtained using two-sided Wilcoxon rank-sum tests and adjusted across the 12 ROC analyses (3 reagents × 4 parameters) using the Bonferroni-Holm method. All statistical analyses were performed in R (version 4.4.0). A two-tailed p value < 0.05 was considered statistically significant after correction for multiple comparisons.

RESULTS

Study population and baseline characteristics

Baseline characteristics of both the included BDUC patients and healthy volunteers are presented in Table 1. A total of 83 BDUC patients and 107 healthy volunteers were included. BDUC patients had a median age of 45 years (IQR 34–56) and were predominantly female (79/83, 95.2%), with a median ISTH-BAT score of 8 (IQR 6–12). Healthy volunteers were younger (median 32 years, IQR 25.5–46) and included a higher proportion of males (35/107, 32.7%). Oral contraceptive use was reported in 1 BDUC patient (1.2%) and 22 healthy volunteers (20.6%). Laboratory parameters, including LTA, were within normal limits in all BDUC patients, consistent with the BDUC diagnosis (LTA data not shown).

Inter- and intra-assay variability in normal pool plasma

All inter- and intra-assay CVs were below 15% (Supplementary table 2).

Thrombin generation: comparison between BDUC patients and healthy volunteers

TGA results for all three trigger reagents are presented in Table 2. With PPP Reagent INT, lag time was significantly prolonged in BDUC patients compared to healthy volunteers

Table 1: All continuous variables: median (IQR), unless otherwise stated. Categorical variables: n (%). Variables not assessed in healthy volunteers are indicated as 'not collected'. (n=BDUC/HV) after a variable name indicates the number of participants with available data when fewer than the full cohort were measured (BDUC n=83; healthy volunteers n=107); when data were only available in one group, only that n is shown. Among healthy volunteers, 14 factor XIII activity results and one free protein S result remained >150% despite dilution up to 1:8 and 3:8, respectively, and were included as right-censored observations in the baseline characteristics. BDUC, bleeding disorder of unknown cause; HV, healthy volunteers; ISTH-BAT, International Society on Thrombosis and Hemostasis Bleeding Assessment Tool; WBC, white blood cell count; PT, prothrombin time; aPTT, activated partial thromboplastin time; VWF, von Willebrand factor.

Characteristic	BDUC (n = 83)	Healthy volunteers (n = 107)
Demographics		
Age (years)	45.0 (34.0–56.0)	32.0 (25.5–46.0)
Female sex, n (%)	79 (95.2%)	72 (67.3%)
Clinical		
Oral contraceptives, n (%)	1 (1.2%)	22 (20.6%)
ISTH-BAT score	8.0 (6.0–12.0)	not collected
Laboratory parameters		
Blood group O/A/B/AB, n	40/33/7/2	not collected
Hemoglobin (mmol/L)	8.40 (8.00–8.80)	not collected
Platelets ($\times 10^9$/L)	265 (220–304)	not collected
WBC ($\times 10^9$/L) (n=82)	5.8 (4.8–6.7)	not collected
PT (s)	10.5 (10.3–10.9)	10.7 (10.3–11.0)
aPTT (s) (n=82/107)	28.0 (27.0–30.0)	23.8 (22.6–25.2)
Fibrinogen (g/L)	3.20 (2.70–3.65)	2.91 (2.55–3.27)
VWF antigen (%) (n=83/98)	102 (76–128)	120.4 (96.3–150.6)
VWF activity (%) (n=83/98)	103 (72–134)	126.5 (96.5–165.3)
Factor II (%)	112 (97–125)	123 (112–134)
Factor V (%)	106 (92–118)	130 (112–147)
Factor VII (%) (n=83/106)	108 (89–122)	120 (98–144)
Factor VIII (%)	123 (104–158)	124 (111–155)
Factor IX (%)	111 (99–128)	112 (100–123)
Factor X (%)	106 (92–120)	124 (111–144)
Factor XI (%)	120 (102–140)	148 (126–150)
Factor XII (%) (n=64/107)	132 (101–159)	127 (96–150)
Factor XIII (%) (n=83/98)	128 (110–150)	123.7 (109.0–142.9)

Characteristic	BDUC (n = 83)	Healthy volunteers (n = 107)
Antithrombin (%) (n=65/107)	112 (105–120)	112 (106–120)
Protein C (%) (n=65/107)	119 (105–133)	107 (100–125)
Free protein S (%) (n=64/98)	111 (98–122)	101.2 (85.1–113.0)

(4.83 [4.33–5.33] vs. 4.33 [4.00–4.67] min; p < 0.001), while ETP, peak height, and velocity index did not differ significantly between groups. With PPP Reagent LOW, no significant differences were observed for any of the four parameters. With PPP Reagent LOW 1:5 PL, lag time was prolonged (6.67 [5.75–7.50] vs. 6.00 [5.33–7.00] min; p = 0.034) and ETP was reduced (1207 [1012–1471] vs. 1357 [1174–1669] nM·min; p = 0.018) in BDUC patients, whereas peak height and velocity index did not reach statistical significance. The mean thrombin generation curves of the BDUC and healthy volunteer groups for each of the three reagents are presented in Figure 2.

Table 2: Values presented as median (IQR) or mean ± SD. Groups were compared using the Mann-Whitney U test, except for velocity index with PPP Reagent INT, which was normally distributed and compared using Welch's t-test. P values were adjusted for multiple testing across 12 simultaneous comparisons (3 reagents × 4 parameters) using the Bonferroni-Holm method. NS, not significant; *, p < 0.05; ***, p < 0.001.

Parameter	n	BDUC patients	n	Healthy volunteers	p value	Sig.
PPP Reagent INT						
Lag time (min)	83	4.83 (4.33–5.33)	107	4.33 (4.00–4.67)	<0.001	***
ETP (nM·min)	83	1876 (1684–2181)	107	2026 (1816–2216)	0.609	NS
Peak height (nM)	83	509 (454–554)	107	515 (482–563)	0.745	NS
Velocity index (nM/min)	83	330 ± 50	107	342 ± 51	0.609	NS
PPP Reagent LOW						
Lag time (min)	83	5.83 (4.83–6.92)	107	5.67 (4.83–6.17)	0.350	NS
ETP (nM·min)	83	1327 (1139–1558)	107	1430 (1218–1660)	0.350	NS
Peak height (nM)	83	153 (118–201)	107	164 (122–190)	1.000	NS
Velocity index (nM/min)	83	30.1 (19.9–46.2)	107	29.8 (19.4–39.5)	1.000	NS
PPP Reagent LOW 1:5 PL						
Lag time (min)	83	6.67 (5.75–7.50)	107	6.00 (5.33–7.00)	0.034	*
ETP (nM·min)	83	1207 (1012–1471)	107	1357 (1174–1669)	0.018	*
Peak height (nM)	83	104 (78.5–145)	107	121 (93.9–153)	0.088	NS
Velocity index (nM/min)	83	14.1 (9.49–23.1)	107	17.0 (12.5–24.2)	0.350	NS

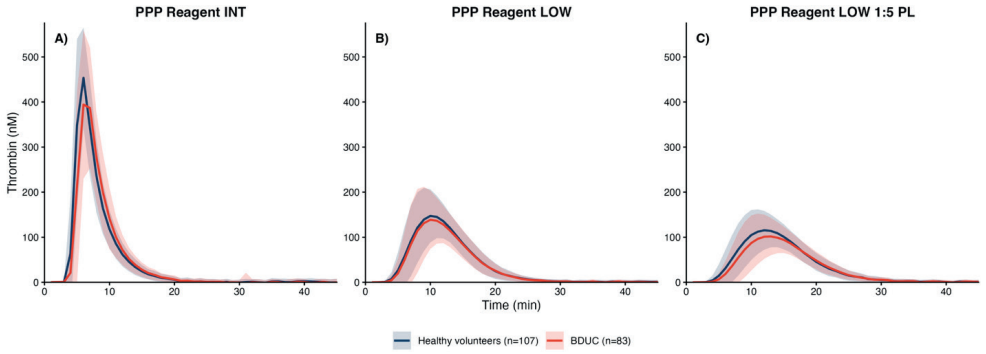


Figure 2: Mean ± standard deviation (SD) thrombin generation curves for BDUC patients (n = 83) and healthy volunteers (n = 107), measured with PPP Reagent INT (A), PPP Reagent LOW (B), and PPP Reagent LOW 1:5 PL (C). Shaded areas represent ±1 SD.

Proportion of abnormal TGA results

The proportions of BDUC patients with abnormal TGA results per parameter and in total are shown in Table 3. With PPP Reagent INT, 10 patients (12.0%) had at least one abnormal result consistent with a bleeding profile, most commonly a prolonged lag time (7.2%) or reduced peak height (4.8%). With PPP Reagent LOW, 10 patients (12.0%) had at least one abnormal result suggestive of a reduced hemostatic profile, predominantly a reduced peak height (8.4%). With PPP Reagent LOW 1:5 PL, 8 patients (9.6%) had at least one abnormal result in the bleeding direction, most commonly a reduced ETP (7.2%).

Results associated with a procoagulant state were observed in a small minority of patients across all reagents. With PPP Reagent INT, 7 patients (8.4%) showed a thrombotic profile, primarily an elevated peak height (7.2%) or elevated ETP (4.8%). With PPP Reagent LOW and PPP Reagent LOW 1:5 PL, 2 patients each (2.4%) had a hypercoagulable characteristic, involving elevated ETP, peak height, or velocity index.

Overall, 28 patients (33.7%) had at least one TGA abnormality in any direction across any reagent, of whom 21 (25.3%) had a bleeding-direction abnormality and 7 (8.4%) a thrombotic-direction abnormality in at least one reagent.

Correlations between TGA parameters and clinical and laboratory variables in BDUC and healthy volunteers

In BDUC patients (n = 83), no significant associations were observed between TGA parameters and age or the ISTH-BAT score. Significant correlations were observed with aPTT, fibrinogen, coagulation factors II, VII, VIII, IX, X, and XI, and protein C (Table 4). In healthy volunteers (n = 107), significant correlations were observed between TGA

parameters and age, aPTT, PT, fibrinogen, VWF activity, coagulation factors II, VII, IX, X, and XI, protein C, and free protein S (Supplementary table 3).

Table 3: Reference ranges (P2.5–P97.5) derived from healthy volunteers. A result was classified as abnormal in the bleeding direction when lag time was prolonged and/or ETP, peak height, or velocity index fell below the lower reference limit. A result was classified as abnormal in the thrombotic direction when lag time was shortened and/or ETP, peak height, or velocity index exceeded the upper reference limit.

Parameter	P2.5	P97.5	n bleeding direction	%	n thrombotic direction	%
PPP Reagent INT						
Lag time (min)	3.28	5.84	6	7.2%	0	0.0%
ETP (nM·min)	1377	2782	0	0.0%	4	4.8%
Peak height (nM)	410	637	4	4.8%	6	7.2%
Velocity index (nM/min)	247	468	2	2.4%	0	0.0%
Any abnormality (≥1 parameter)			**10**	**12.0%**	**7**	**8.4%**
PPP Reagent LOW						
Lag time (min)	3.62	8.88	5	6.0%	0	0.0%
ETP (nM·min)	852	2491	1	1.2%	2	2.4%
Peak height (nM)	80.8	344	7	8.4%	2	2.4%
Velocity index (nM/min)	11.3	114	5	6.0%	2	2.4%
Any abnormality (≥1 parameter)			**10**	**12.0%**	**2**	**2.4%**
PPP Reagent LOW 1:5 PL						
Lag time (min)	3.90	9.88	1	1.2%	0	0.0%
ETP (nM·min)	876	2384	6	7.2%	2	2.4%
Peak height (nM)	56.6	251	2	2.4%	2	2.4%
Velocity index (nM/min)	5.82	63.4	3	3.6%	2	2.4%
Any abnormality (≥1 parameter)			**8**	**9.6%**	**2**	**2.4%**

Table 4: Spearman rank correlation (r) between 12 TGA parameters and 22 clinical/laboratory variables in BDUC patients (n = 83). Bonferroni-Holm correction over all 264 pairs; 32 pairs were significant. Only significant correlations are shown. Green = positive correlation (r > 0); red = negative correlation (r < 0). *** p < 0.001, ** p < 0.01, * p < 0.05.

Reagent	TGA parameter	Clinical variable	n	r	p value	Sig.
PPP Reagent INT						
	Lag time	aPTT (s)	82	**0.740**	**<0.001**	***
	Peak height	Factor II (%)	83	**0.700**	**<0.001**	***
	Peak height	Factor X (%)	83	**0.598**	**<0.001**	***
	ETP	Factor II (%)	83	**0.575**	**<0.001**	***
	Velocity index	Factor II (%)	83	**0.541**	**<0.001**	***
	ETP	Factor X (%)	83	**0.522**	<0.001	***
	Peak height	Factor IX (%)	83	**0.474**	**0.001**	**
	Velocity index	Factor X (%)	83	**0.467**	**0.002**	**
	Velocity index	Factor IX (%)	83	0.440	0.008	**
	Peak height	Factor XI (%)	83	**0.435**	**0.010**	**
	Lag time	Factor VIII (%)	83	**−0.423**	**0.016**	*
	Velocity index	Protein C (%)	65	**0.471**	**0.018**	*
	Peak height	Factor VII (%)	83	**0.411**	**0.027**	*
	ETP	Factor IX (%)	83	**0.402**	**0.039**	*
PPP Reagent LOW						
	Velocity index	aPTT (s)	82	**−0.585**	**<0.001**	***
	Peak height	aPTT (s)	82	**−0.578**	**<0.001**	***
	Velocity index	Factor VIII (%)	83	**0.501**	**<0.001**	***
	Peak height	Factor VIII (%)	83	**0.491**	**<0.001**	***
	ETP	aPTT (s)	82	**−0.478**	**0.001**	**
	Lag time	Fibrinogen (g/L)	83	**0.466**	**0.002**	**
	ETP	Factor VIII (%)	83	**0.419**	**0.019**	*
	Velocity index	Factor IX (%)	83	**0.414**	**0.023**	*
	Peak height	Factor IX (%)	83	**0.401**	0.040	*
PPP Reagent LOW 1:5 PL						
	Velocity index	aPTT (s)	82	**−0.477**	**0.001**	**
	Velocity index	Factor VIII (%)	83	**0.468**	**0.002**	**
	Peak height	aPTT (s)	82	**−0.466**	**0.003**	**
	Peak height	Factor VIII (%)	83	**0.454**	**0.004**	**
	Lag time	Fibrinogen (g/L)	83	**0.452**	**0.004**	**
	Velocity index	Factor IX (%)	83	**0.431**	**0.011**	*
	ETP	aPTT (s)	82	**−0.423**	**0.018**	*

Reagent	TGA parameter	Clinical variable	n	r	p value	Sig.
	Peak height	Factor IX (%)	83	**0.416**	**0.022**	*
	ETP	Factor VIII (%)	83	**0.403**	**0.038**	*

Correlations were also computed in healthy volunteers (n = 107) and compared with the corresponding correlations in BDUC patients. Of 216 tested pairs, one differed significantly: the correlation between lag time (PPP Reagent INT) and aPTT was substantially stronger in BDUC patients than in healthy volunteers (r = 0.74 vs. r = 0.16; p < 0.001) (Table 5).

Table 5: Spearman rank correlation (r) computed separately in BDUC (n = 83) and HV (healthy volunteers; n = 107) for 18 clinical variables available in both groups. Correlations compared using Fisher's z-transformation test for independent samples. Bonferroni-Holm correction over all 216 pairs (12 TGA parameters × 18 clinical variables); one pair was significantly different. Only the significantly different correlation is shown. *** p < 0.001.

Reagent	TGA parameter	Clinical variable	r BDUC	n BDUC	r HV	n HV	z statistic	p value	Sig.
PPP Reagent INT									
	Lag time	aPTT (s)	0.740	82	0.155	107	5.318	<0.001	***

Discriminative ability of TGA parameters

ROC analysis was performed to assess the ability of TGA parameters to discriminate between BDUC patients and healthy volunteers (Table 6). Lag time with PPP Reagent INT showed the strongest discriminative ability (AUC 0.691; 95% CI 0.616–0.767; p < 0.001). With PPP Reagent LOW 1:5 PL, AUCs ranged from 0.583 to 0.634, although only lag time and ETP retained statistical significance after correction for multiple testing (lag time: AUC 0.624, p = 0.034; ETP: AUC 0.634, p = 0.018; peak height: AUC 0.609, p = 0.088; velocity index: AUC 0.583, p = 0.350). With PPP Reagent LOW, none of the four parameters reached significance after correction (lag time AUC 0.585, p = 0.350). With PPP Reagent INT, only lag time reached significance. The resulting ROC curves are shown in Supplementary figures 1-3.

Table 6: Areas under the curve (AUCs) with 95% confidence intervals were calculated using the DeLong method. P values for the null hypothesis of AUC = 0.5 were obtained using two-sided Wilcoxon rank-sum tests and adjusted across the 12 ROC analyses (3 reagents × 4 parameters) using the Bonferroni-Holm method. CI, confidence interval; NS, not significant; *, p < 0.05; ***, p < 0.001.

Reagent	Parameter	AUC	95% CI	p value	Sig.
PPP Reagent INT					
	Lag time (min)	0.691	0.616–0.767	<0.001	***
	ETP (nM·min)	0.565	0.481–0.649	0.621	NS
	Peak height (nM)	0.549	0.464–0.633	0.745	NS
	Velocity index (nM/min)	0.564	0.481–0.646	0.621	NS
PPP Reagent LOW					
	Lag time (min)	0.585	0.502–0.668	0.350	NS
	ETP (nM·min)	0.583	0.500–0.665	0.350	NS
	Peak height (nM)	0.525	0.441–0.610	1.000	NS
	Velocity index (nM/min)	0.508	0.423–0.592	1.000	NS
PPP Reagent LOW 1:5 PL					
	Lag time (min)	0.624	0.544–0.704	0.034	*
	ETP (nM·min)	0.634	0.553–0.714	0.018	*
	Peak height (nM)	0.609	0.527–0.692	0.088	NS
	Velocity index (nM/min)	0.583	0.500–0.667	0.350	NS

DISCUSSION

This study evaluated whether TGA can identify deviations from reference values in patients with BDUC and compared the informativeness of three trigger reagents, including a novel intrinsic trigger. Compared with healthy volunteers, BDUC patients showed a significantly prolonged lag time with PPP Reagent INT and PPP Reagent LOW 1:5 PL, whereas no significant between-group differences were observed with PPP Reagent LOW. With PPP Reagent LOW 1:5 PL, ETP was additionally reduced. Across the three trigger reagents, 21/83 (25%) patients had at least one TGA abnormality in the bleeding direction. These findings are further supported by a recent study by Dreier et al. (2026), who demonstrated impaired TGA as a reproducible feature of BDUC in an independent cohort using the fully automated Ceveron S100 platform (16).

The secondary aim was to identify the most informative trigger reagent for detecting TGA abnormalities in the bleeding direction. No single reagent consistently outperformed the others, as the proportions of patients with abnormal TGA results were comparable across reagents. Notably, PPP Reagent LOW did not show significant differences at the group level, despite identifying at least one bleeding-direction abnormality in 12.0% of individual patients. This illustrates that group-level comparisons and classification based on reference intervals capture different aspects of TGA abnormalities. The abnormalities detected also differed according to the trigger used. PPP Reagent INT predominantly identified a prolonged lag time, whereas PPP Reagent LOW 1:5 PL more frequently detected a reduced ETP. These findings suggest that different trigger reagents may capture distinct aspects of the hemostatic disturbance in BDUC rather than differing simply in overall sensitivity. This observation is consistent with previous studies showing that the abnormalities detected by TGA in BDUC depend strongly on assay conditions and trigger composition (16, 21).

Our findings align closely with those of Dreier et al. (2026), who measured TGA using the fully automated Ceveron S100 analyzer in 62 BDUC patients and 22 age- and sex-matched healthy controls (16). Despite methodological differences, including the use of a different platform (automated Ceveron S100 versus our semi-automated CAT on a Fluoroskan Ascent Fluorimeter), calibration (CAT method for inner filter effect, substrate depletion and plasma color), different trigger reagents, and different control populations, both studies identified impaired thrombin generation in BDUC patients. Dreier et al. reported impaired peak thrombin (peak height; maximum thrombin concentration), velocity index, and ETP in addition to a prolonged lag time, particularly with the very low TF reagent, and demonstrated cross-validated ROC AUCs of 0.74 and 0.76 using a multivariate approach incorporating all TGA parameters simultaneously. In our study, lag time showed the strongest univariate discriminative ability (AUC 0.691 with PPP Reagent

INT), while PPP Reagent LOW 1:5 PL showed the broadest discriminative pattern across all four parameters, although only lag time and ETP retained statistical significance after correction for multiple testing. Even for these parameters, substantial overlap between the distributions of BDUC patients and healthy volunteers remained, as reflected by the modest AUC values. The convergence of findings across independent cohorts, different platforms, and different reagent types provides important evidence for the reproducibility of impaired thrombin generation as a characteristic feature of BDUC across multiple independent studies (13-16).

Consistent with Dreier et al. (2026), no correlation was observed between TGA parameters and the ISTH-BAT score in our cohort (16). This may be explained by the fact that the ISTH-BAT score is strongly influenced by lifetime exposure to hemostatic challenges. As a result, individuals who have not yet undergone major surgeries, dental procedures, or other significant bleeding challenges may have low scores despite the presence of an underlying hemostatic defect, potentially obscuring associations with laboratory phenotypes. Alternatively, the bleeding tendency in BDUC may be influenced by factors beyond thrombin generation capacity, such as the vessel wall or the fibrinolytic system. Indeed, a previous study demonstrated that 21% of BDUC patients from the ProBe-AHP cohort had a hyperfibrinolytic profile on tPA-ROTEM, whereas 58% showed a normal fibrinolytic profile, further underscoring the clinical heterogeneity of this population (22).

The pattern of correlations with coagulation factor levels and aPTT observed in BDUC patients is in accordance with findings by Dielis et al. (2008), who identified intrinsic and common pathway factors as key determinants of TGA parameters in a healthy population (23). Associations between thrombin generation parameters and factors II, VIII, IX, and X were among the most prominent findings, although their pattern differed according to the trigger reagent used. These findings broadly support intrinsic and common pathway factors as important determinants of thrombin generation. Notably, the correlation between lag time (PPP Reagent INT) and aPTT was significantly stronger in BDUC patients than in healthy volunteers ($r = 0.74$ vs. $r = 0.16$), suggesting that in BDUC patients, aPTT more strongly reflects delayed initiation of thrombin generation, even though aPTT values remained within the reference range in this population. This was further supported by inverse correlations between aPTT and propagation parameters (velocity index, peak height, and ETP) across extrinsic triggers (PPP Reagent LOW and PPP Reagent LOW 1:5 PL), suggesting that prolonged clotting times are associated not only with delayed initiation, but also with reduced thrombin generation during the propagation phase. However, not all observed associations fit within this established framework. Two additional correlations merit brief discussion: a positive correlation between protein C and velocity index (PPP Reagent INT, $r = 0.47$), which likely reflects co-regulation of hepatic pro- and anticoagulant factor synthesis rather than a direct anticoagulant effect, and a positive association

between fibrinogen and lag time across two reagents (r ≈ 0.45–0.47; PPP Reagent LOW and PPP Reagent LOW 1:5 PL), which is consistent with Dielis et al. (2008), who also identified fibrinogen as a determinant of lag time and suggested that fibrin(ogen)-mediated inhibition of thrombin-dependent FVIII activation may contribute to this relationship (23).

This study has several limitations. The absence of longitudinal TGA measurements limits the ability to assess intraindividual variability over time and to evaluate the predictive value of TGA parameters for future bleeding risk. As BDUC patients were selected from the ProBe-AHP cohort based on an elevated ISTH-BAT score, selection bias cannot be excluded, and the ISTH-BAT itself carries known limitations as a diagnostic tool (24). Furthermore, the healthy volunteer group was not matched for age, sex, or oral contraceptive use, which may have influenced reference range estimates and between-group comparisons. However, no significant associations between age or sex and TGA parameters were observed within the BDUC cohort, suggesting that demographic differences are unlikely to fully explain the observed findings.

Future research should investigate the pathophysiological mechanisms underlying impaired thrombin generation in BDUC. Longitudinal studies are needed to determine whether TGA abnormalities are stable over time and whether they predict bleeding risk during hemostatic challenges such as surgery or childbirth. TGA using platelet-rich plasma, which incorporates platelet-dependent contributions to thrombin generation, may identify additional abnormalities relevant to the bleeding phenotype in this population. The potential role of natural anticoagulants, such as elevated tissue factor pathway inhibitor or activated protein C levels, as contributors to impaired TGA in BDUC also warrants further investigation (25).

In conclusion, our results confirm that TGA can detect deviations from reference values in a subset of BDUC patients, predominantly a prolonged lag time reflecting delayed initiation of thrombin generation. PPP Reagent LOW 1:5 PL provided the broadest discriminative profile across all four TGA parameters, and lag time with PPP Reagent INT demonstrated the strongest individual discriminative ability (AUC 0.691). These findings are consistent with the reproducibility of impaired TGA in BDUC as reported by Dreier et al. (2026) and collectively suggest that impaired thrombin generation may represent a characteristic feature of the hemostatic disturbance in a proportion of BDUC patients. The clinical utility of TGA in BDUC, including its potential to guide therapeutic decisions, remains to be established in prospective studies.

SUPPLEMENTALS

Supplementary table 1: Overview of hemostasis tests performed in patients from the ProBe-AHP study. PT, prothrombin time; aPTT, activated partial thromboplastin time; VWF, von Willebrand factor; PFA-200, platelet function analyzer 200; LTA, light transmission aggregometry; ATP-release, adenosine triphosphate release.

Category	Tests performed
Screening hemostasis tests	Complete blood count, PT, aPTT, fibrinogen
Von Willebrand tests	VWF activity and VWF antigen
Coagulation factors	FII, FV, FVII, FVIII, FIX, FX, FXI, FXIII
Platelet function tests	PFA-200, LTA, ATP-release

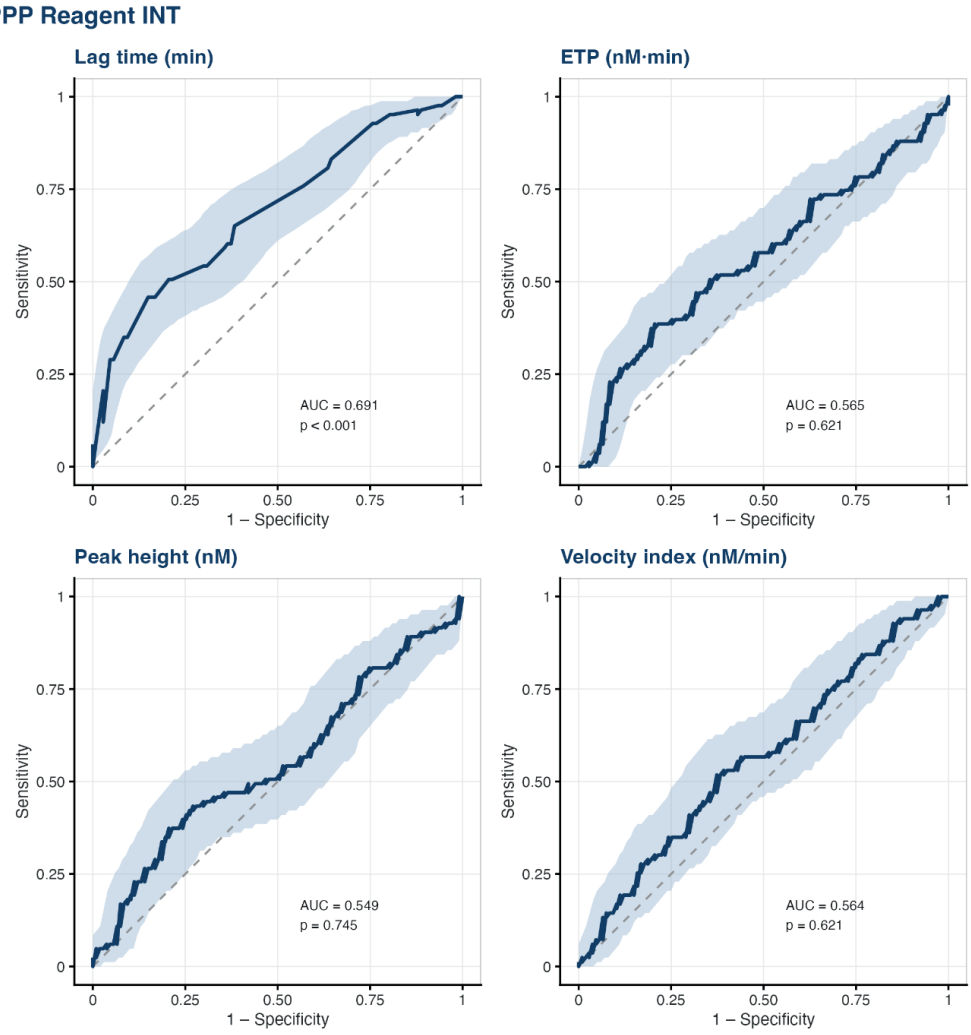
Supplementary table 2: Inter- and intra-assay CVs of the trigger reagents PPP Reagent INT, PPP Reagent LOW and PPP Reagent LOW 1:5 PL were determined in normal pool plasma from measurements performed in duplicate or triplicate, depending on plasma availability, on 26 consecutive days.

Parameter	PPP Reagent INT	PPP Reagent LOW	PPP Reagent LOW 1:5 PL
Inter-assay coefficient of variation (%)			
Lag time	6.10	6.36	7.93
ETP	5.19	3.26	8.48
Peak height	3.91	5.58	8.75
Velocity index	8.37	12.26	13.11
Intra-assay coefficient of variation (%)			
Lag time	3.17	14.47	2.73
ETP	2.24	12.20	4.26
Peak height	1.98	12.26	6.65
Velocity index	10.16	12.71	12.08

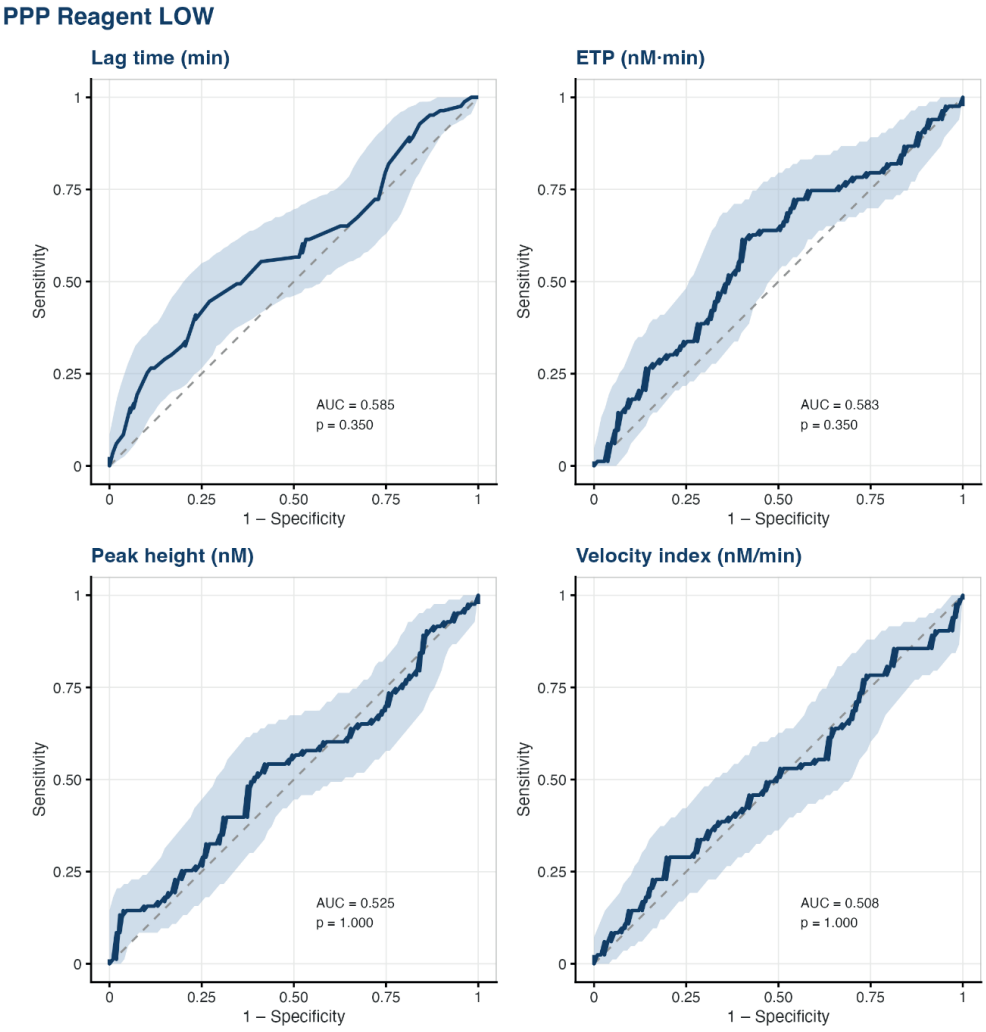
Supplementary table 3: Spearman rank correlation between 12 TGA parameters and 18 clinical/laboratory variables available in healthy volunteers (n = 107). Bonferroni-Holm correction over all 216 pairs; 55 pairs were significant. Only significant correlations are shown. Among healthy volunteers, 14/98 factor XIII activity results and 1/98 free protein S result remained >150% despite dilution up to 1:8 and 3:8, respectively; these right-censored results were excluded from the correlation analyses, which used exact numeric results only (factor XIII, n = 84; free protein S, n = 97). Green = positive correlation; red = negative correlation. *** p < 0.001, ** p < 0.01, * p < 0.05.

Reagent	TGA parameter	Clinical variable	n	r (Spearman)	P-value (Holm)	Sig.
PPP Reagent INT						
	Peak height	Factor II (%)	107	**0.852**	**<0.001**	***
	Velocity index	Factor II (%)	107	**0.707**	**<0.001**	***
	ETP	Factor II (%)	107	**0.652**	**<0.001**	***
	Peak height	Factor X (%)	107	**0.645**	**<0.001**	***
	Velocity index	Factor X (%)	107	**0.633**	**<0.001**	***
	Peak height	Fibrinogen (g/L)	107	**0.548**	**<0.001**	***
	Peak height	Factor IX (%)	107	**0.539**	**<0.001**	***
	Velocity index	Factor IX (%)	107	**0.507**	**<0.001**	***
	ETP	Factor X (%)	107	**0.490**	**<0.001**	***
	ETP	Fibrinogen (g/L)	107	**0.472**	**<0.001**	***
	ETP	PT (s)	107	**-0.453**	**<0.001**	***
	ETP	Factor IX (%)	107	**0.449**	**<0.001**	***
	Peak height	Protein C (%)	107	**0.448**	**<0.001**	***
	Velocity index	Fibrinogen (g/L)	107	**0.448**	**<0.001**	***
	Velocity index	Free protein S (%)	97	**0.426**	**0.002**	**
	Peak height	PT (s)	107	**-0.422**	**0.001**	**
	Peak height	Factor XI (%)	107	**0.414**	**0.002**	**
	Peak height	Factor VII (%)	106	**0.411**	**0.002**	**
	Peak height	Free protein S (%)	97	**0.385**	**0.017**	*
	Velocity index	Protein C (%)	107	**0.383**	**0.008**	**
	ETP	Factor VII (%)	106	**0.368**	**0.018**	*
	Velocity index	Factor XI (%)	107	**0.357**	**0.027**	*
	Velocity index	PT (s)	107	**-0.344**	**0.047**	*
PPP Reagent LOW						
	ETP	PT (s)	107	**-0.510**	**<0.001**	***
	ETP	Factor II (%)	107	**0.485**	**<0.001**	***
	Peak height	PT (s)	107	**-0.460**	**<0.001**	***

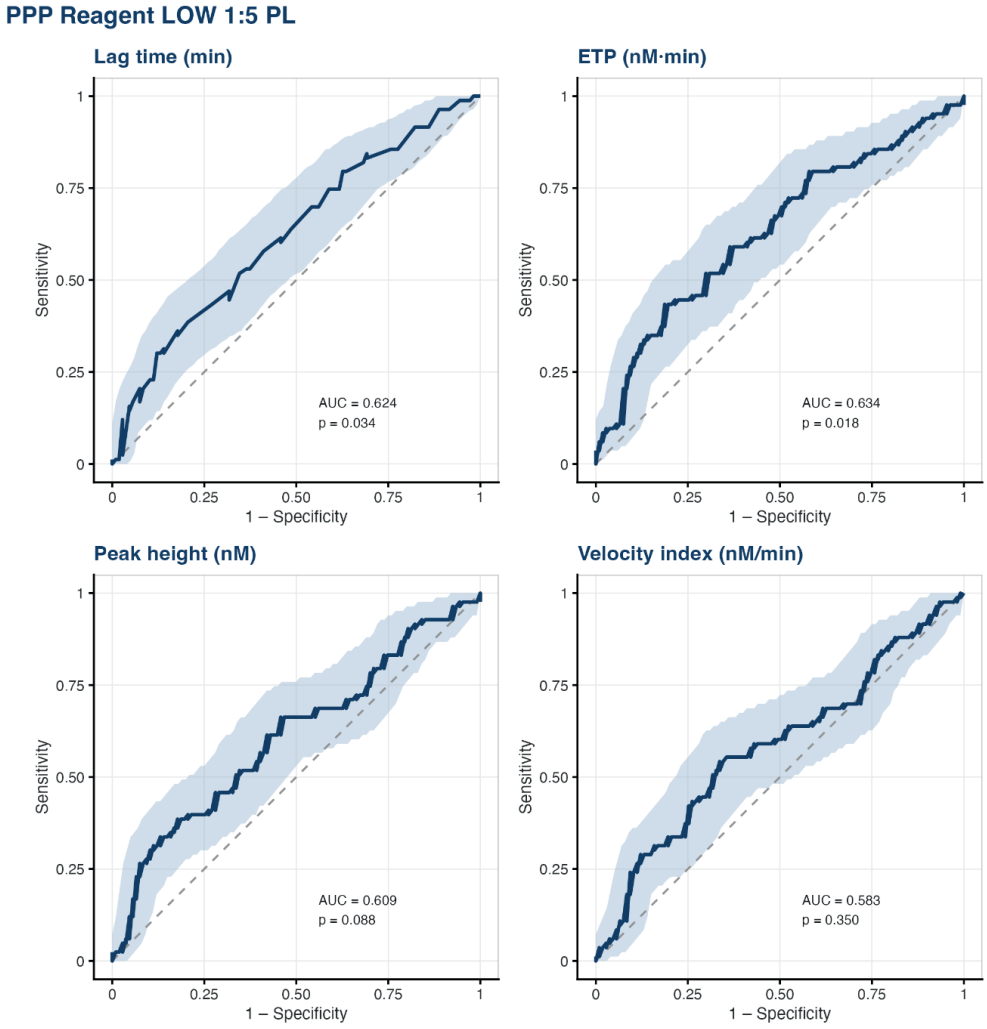
Reagent	TGA parameter	Clinical variable	n	r (Spearman)	P-value (Holm)	Sig.
	Lag time	Free protein S (%)	97	**0.450**	**<0.001**	***
	Peak height	Factor X (%)	107	**0.444**	**<0.001**	***
	Velocity index	aPTT (s)	107	**-0.427**	**<0.001**	***
	Velocity index	Factor X (%)	107	**0.421**	**0.001**	**
	Velocity index	PT (s)	107	**-0.410**	**0.002**	**
	ETP	Factor X (%)	107	**0.407**	**0.002**	**
	Peak height	Factor II (%)	107	**0.406**	**0.003**	**
	Peak height	aPTT (s)	107	**-0.404**	**0.003**	**
	Peak height	Factor IX (%)	107	**0.401**	**0.003**	**
	Velocity index	Factor IX (%)	107	**0.397**	**0.004**	**
	ETP	Fibrinogen (g/L)	107	**0.378**	**0.010**	*
	ETP	Factor IX (%)	107	**0.359**	**0.025**	*
	Velocity index	VWF activity (%)	98	**0.359**	**0.047**	*
	Velocity index	Factor II (%)	107	**0.346**	**0.043**	*
	Lag time	Age (years)	107	**0.343**	**0.048**	*
PPP Reagent LOW 1:5 PL						
	ETP	PT (s)	107	**-0.481**	**<0.001**	***
	Peak height	PT (s)	107	**-0.477**	**<0.001**	***
	Velocity index	PT (s)	107	**-0.453**	**<0.001**	***
	Velocity index	Factor X (%)	107	**0.440**	**<0.001**	***
	Peak height	Factor X (%)	107	**0.440**	**<0.001**	***
	ETP	Factor II (%)	107	**0.424**	**0.001**	**
	Lag time	Free protein S (%)	97	**0.415**	**0.004**	**
	Peak height	Factor IX (%)	107	**0.403**	**0.003**	**
	Velocity index	Factor IX (%)	107	**0.400**	**0.004**	**
	ETP	Factor X (%)	107	**0.378**	**0.010**	*
	Peak height	Factor II (%)	107	**0.365**	**0.019**	*
	Lag time	Age (years)	107	**0.348**	**0.039**	*
	Velocity index	Factor VII (%)	106	**0.345**	**0.048**	*
	Velocity index	aPTT (s)	107	**-0.342**	**0.0499**	*



Supplementary figure 1: Receiver operating characteristic (ROC) curves for thrombin generation parameters measured with PPP Reagent INT in BDUC patients (n = 83) versus healthy volunteers (n = 107). Each panel shows sensitivity versus 1 – specificity for one of the four thrombin generation parameters: lag time, endogenous thrombin potential (ETP), peak height, and velocity index. The area under the curve (AUC) and the p value obtained from a two-sided Wilcoxon rank-sum test and adjusted across the 12 ROC analyses using the Bonferroni–Holm method are shown within each panel. The 95% confidence interval for the AUC, calculated using the DeLong method, is reported in Table 6. The shaded area represents the 95% confidence band.



Supplementary figure 2: Receiver operating characteristic (ROC) curves for thrombin generation parameters measured with PPP Reagent LOW in BDUC patients (n = 83) versus healthy volunteers (n = 107). Each panel shows sensitivity versus 1 – specificity for one of the four thrombin generation parameters: lag time, endogenous thrombin potential (ETP), peak height, and velocity index. The area under the curve (AUC) and the p value obtained from a two-sided Wilcoxon rank-sum test and adjusted across the 12 ROC analyses using the Bonferroni–Holm method are shown within each panel. The 95% confidence interval for the AUC, calculated using the DeLong method, is reported in Table 6. The shaded area represents the 95% confidence band.



Supplementary figure 3: Receiver operating characteristic (ROC) curves for thrombin generation parameters measured with PPP Reagent LOW 1:5 PL in BDUC patients (n = 83) versus healthy volunteers (n = 107). Each panel shows sensitivity versus 1 – specificity for one of the four thrombin generation parameters: lag time, endogenous thrombin potential (ETP), peak height, and velocity index. The area under the curve (AUC) and the p value obtained from a two-sided Wilcoxon rank-sum test and adjusted across the 12 ROC analyses using the Bonferroni–Holm method are shown within each panel. The 95% confidence interval for the AUC, calculated using the DeLong method, is reported in Table 6. The shaded area represents the 95% confidence band.

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CHAPTER 7

DIAGNOSTIC VALUE OF PLATELET FUNCTION TESTS, INCLUDING THE FLOW CYTOMETRY BASED PLATELET ACTIVATION TEST (PACT), IN PATIENTS WITH A SUSPECTED CONGENITAL PLATELET FUNCTION DISORDER.

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Submitted

ABSTRACT

Background: The diagnostic workup of mild-to-moderate inherited platelet disorders (IPD) in patients who present with mucocutaneous bleeding symptoms is challenging and requires labor-intensive platelet function tests. Light transmission aggregometry, the gold standard test, lacks sensitivity for mild IPDs, necessitating additional platelet function assays.

Aim: To determine the diagnostic yield of LTA, platelet ADP content, flow cytometry based receptor expression and platelet activation test (PACT) in patients with a suspected primary hemostasis defect.

Methods: Data from the 'Thrombocytopathy in the Netherlands (TiN)' cohort were used, which included patients with a suspected IPD in whom Von Willebrand disease was excluded. Diagnostic workup included complete blood count, platelet morphology, LTA, platelet nucleotide content tests, flow cytometry-based receptor expression and PACT. The bleeding phenotype was assessed with the ISTH-BAT score. Medical ethics committee approval was obtained and all participants provided informed consent.

Results: 158 patients with an elevated ISTH-BAT score were included. In 12/158 patients, LTA and in 13/158 platelet ADP content could not be performed due to low platelet count or technical defects. LTA showed abnormal results in 66/146 patients and had a diagnostic yield of 45%. Platelet ADP content had an additional diagnostic yield of 8%, flow cytometry based receptor expression had an additional diagnostic yield of 3% and PACT had an additional diagnostic yield of 19%. The remaining 67 patients with normal platelet function tests were classified as having a bleeding disorder of unknown cause (BDUC).

Conclusion: In a group of 158 patients with an elevated ISTH-BAT score and a suspected IPD, the combination of LTA, platelet ADP content, flow cytometry based receptor expression and PACT had a combined diagnostic yield of 58%.

INTRODUCTION

Platelets play an essential role in maintaining hemostasis, particularly within the primary hemostasis pathway. In case of vascular damage, platelets are immediately able to adhere to the extracellular matrix components on the site of injury. This triggers platelet activation and aggregation, ultimately resulting in the formation of a firm platelet plug that prevents further bleeding (1).

Patients suspected of having a primary hemostasis pathway defect often present with a characteristic bleeding pattern, including symptoms of epistaxis, spontaneous hematomas, oral cavity bleeds, heavy menstrual bleeding (HMB) and bleeding during or after hemostatic challenges such as surgery, dental extractions or childbirth (2). A bleeding assessment tool (BAT) can be used to quantify the bleeding phenotype. The most commonly used is the 'International Society on Thrombosis and Hemostasis (ISTH)-BAT' (3). While BATs can be helpful in identifying and structuring a patients bleeding phenotype, they also have limitations, as a low BAT-score does not exclude a mild bleeding disorder (MBD) (4, 5). Furthermore, it remains challenging to distinguish patients with different MBD's based on their bleeding phenotype alone, as there is a lot of overlap between for example patients with Von Willebrand's disease (VWD) and patients with mild to moderate inherited platelet defects (IPDs) (6).

In recent years, increasing attention has been drawn to a group of patients within the MBD group, who are classified as having a bleeding disorder of unknown cause (BDUC). These patients account for approximately 40-70% of all patients referred to a hemostasis specialist for evaluation of an increased bleeding tendency. BDUC patients have a clinically relevant increased bleeding tendency without abnormalities in a set of laboratory hemostasis tests (7-9). It is a diagnosis by exclusion, meaning that all known bleeding disorders and other causes for bleeding must be ruled out. This process requires adequate platelet function diagnostics, amongst others.

The current gold standard platelet function test is light transmission aggregometry (LTA). However, this assay has a poor sensitivity for diagnosis of platelet storage pool disease (SPD) and/or mild platelet function defects (PFD's) (10-12). Therefore, supplementary assays are needed to increase the diagnostic sensitivity, including an assay to diagnose patients with storage pool deficiency, e.g. measurement of ADP and ATP content in platelet lysates (13, 14). Platelet phenotyping and measurement of platelet reactivity with flow cytometry was also suggested to have additive value when measured alongside LTA (15).

In this study, we evaluated the diagnostic yield of the gold standard LTA in combination with additional platelet function assays, including platelet ADP content, flow cytometry-

based receptor expression and platelet reactivity in patients with a suspected primary hemostasis defect.

METHODS

Study population

Data from the thrombocytopathy in the Netherlands (TiN) study (METC 15-597) were used. The TiN study is a nationwide cross-sectional study including patients with a confirmed or suspected inherited platelet disorder (IPD). Inclusion criteria were (i) exclusion of von Willebrand disease (normal vWF:Rco activity levels) and coagulation factor deficiencies (normal PT and aPTT), (ii) previous IPD diagnosis, (iii) previous abnormal platelet count or function test results or (iv) presence of a predominantly mucocutaneous bleeding tendency, compatible with a platelet function disorder. Patients using antiplatelet therapy were excluded. A total of 195 patients were included in the TiN cohort. For this study, patients with a normal ISTH-BAT score (n=33) and patients with an acquired platelet function disorder (n=4) were excluded, resulting in a final study population of n=158. IPD was defined as a bleeding tendency (ISTH-BAT ≥6 women and ≥4 in men) in patients with a normal APTT, PT, VWF:Ag and VWF:Act in whom a platelet function defect was detected. BDUC was defined as a bleeding tendency (ISTH-BAT ≥6 women and ≥4 in men) in patients with a platelet count>100 G/L, normal platelet function test results, normal PT and APTT and normal VWF:Ag and VWF:Act results. All participating patients and healthy volunteers gave written informed consent according to the Helsinki declaration.

Blood collection

Peripheral venous blood was collected from included patients and controls through venipuncture in 3.2% sodium citrate Vacutainer tubes (BD Biosciences). Whole blood was used to perform flow cytometry assays. The remainder of the blood was processed into platelet rich plasma (PRP) and platelet poor plasma (PPP) for the other laboratory tests. PRP was obtained by centrifugation of whole blood at 160 G for 15 minutes at 20 ℃. PPP was obtained by centrifugation of whole blood at 2000 G for 10 minutes. Blood was processed within 1-6 hours after collection.

Bleeding phenotype, laboratory tests and diagnostic criteria

The bleeding phenotype was assessed using the ISTH-BAT score. An ISTH-BAT score of ≥4 in men and ≥6 in women was considered abnormal. Platelet count, aggregation in response to four agonists (ADP (2.5 and 5.0 μM), arachidonic acid, collagen and ristocetin) in the LTA, platelet ADP and ATP content and surface receptor expression with flow cytometry were performed in included patients. Additionally, the Flowcytometric Platelet Activation Test (PACT) was also performed. In supplementary Table S1 the diagnostic criteria for a

specific platelet function disorder were described. Detailed laboratory test methods are described in more detail below.

Laboratory test methods

LTA

The PAP-8E platelet aggregometer (Sysmex, Etten-Leur, Netherlands) was used to perform LTA within 3 hours after blood collection. Citrated whole blood was centrifuged at 160 g for 15 min at 20°C to obtain PRP. The remaining blood was centrifuged at 2000 g for 15 min at 20°C to obtain PPP and this was used to adjust platelet counts to 250 x 10^9 platelets/L^{-1}. LTA data were not obtained when platelet count was ≤75 x 10^9 platelets/L. Different agonists were used to induce aggregation: ADP (5.0 µM), Horm collagen (4.0 µg/mL), arachidonic acid (1.5 mM) or ristocetin (1.0 mg/mL). Samples were stirred at 900 rpm and aggregation traces were recorded for 15 min and the maximal amplitude (MA) of the aggregation curve was evaluated. Cut-off levels for differentiation between normal and abnormal responses were based on the 2.5th percentile of the FA of 58 healthy controls for each agonist. An abnormal LTA test result was defined as 1 or more abnormal aggregation responses.

ATP/ADP content

One milliliter PRP with a platelet count between 100 and 250 x 10^9/L was diluted 1:3 (v:v) in ice cold 86.4% ethanol, 10mM EDTA, pH 7.4. Platelets were lysed by vortex and one freeze/thaw cycle and samples were stored at -80°C until further processing. Platelet lysates were split into two fractions. The first fraction was incubated with PEP-PK (95 µM phosphoenolpyruvate [PEP] and 25 µg/mL pyruvate kinase [PK] in 0.2 M Tris-Maleate, 10 mM KCl, 15 mM $MgSO_4$, pH 7.4) and the second fraction was incubated with heat-inactivated PEP-PK at 37°C for 15 minutes to convert all ADP to ATP. Reactions were stopped by heating the samples for 10 minutes at 80°C. ATP levels in both fractions were determined with the ATPLite 1 step kit (Perkin Elmer) on a spectramax L luminometer (Molecular Devices) according to the protocol of the manufacturer. ATP levels were derived from an ATP calibration curve. ADP concentrations were calculated by subtracting the ATP concentration of the second fraction from the first. ADP levels were expressed in µmol/10^{11} platelets. Dense granule deficiency was diagnosed when the ADP content was lower than 1.4 µmol/10^{11} platelets, based on the 2.5th percentile of 49 healthy volunteers.

Flow cytometry

Citrated whole blood was brought to a temperature of 37°C for 10 min. Subsequently, 5 µL of whole blood was added to 50 µL HEPES-buffered saline (HBS; (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 150 mM NaCl, 1 mM MgSO4, 5 mM KCl, pH 7.4) with fluorescent antibodies with or without platelet agonists and incubated at 37° C for 10 minutes. For quantification of platelet reactivity towards agonists with the platelet activation test (PACT), the following agonists were used: 25 µM protease activated receptor

(PAR)-1 activating peptide (AP) SFLLRN (Bachem), 250 µM PAR-4 AP AYPGKV (Leiden University Medical Center peptide facility), 60 µM adenosine diphosphate (ADP) (Sigma, 01897), 1 µg/ml CRP-XL, 5 µm U-46619 (Cayman chemical, 16450) 1 mg/ml ristocetin (American Biochemical and Pharmaceuticals Ltd; Marlton, NJ, USA). P-selectin expression was quantified with AlexaFluor (AF)647-labeled anti-P-selectin single domain antibody (sdAb) clone B10.6 (7.5 µg/ml), fibrinogen binding with AF488-labeled anti-fibrinogen sdAb clone C3 (25 µg/ml) and VWF binding with AF488-labeled anti-VWF CK-domain sdAb clone sVWF (7.5 µg/ml). R-phycoerythrin (R-PE)-labeled anti-GPIbα sdAb clone 17 (15 µg/ml) was used for gating. AF488- or AF647-labeled anti-azodye RR6 sdAb R2 was used as isotype control for fibrinogen binding and p-selectin expression, respectively. Platelet receptor expression was quantified with FITC conjugated anti-CD49b (clone AK7; BD Pharmingen), R-PE conjugated anti-CD41a (clone HIP8; BD pharmingen) and eFluor660-conjugated anti-GPVI (clone HY101; eBioscience). Samples were fixated with 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH2PO4, 1.15 mM KH2PO4, 10.2 mM Na2HPO4, 4 mM EDTA, 1.11% formaldehyde (pH 6.8) in the dark at RT for 20 minutes. After fixation, samples were diluted 1:1 (v/v) in HBS and analyzed on a BD FACSCanto II Flow Cytometer. Platelets were identified based on forward and side scatter, as well as GPIbα expression. Data are expressed as median fluorescent intensity (MFI).

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 29 and R studios 2024. Continuous variables were presented as either mean ± standard deviation (SD) for normally distributed data or median [interquartile range (IQR)] for non-parametrical data. Categorical variables were presented using counts and percentages.

RESULTS

Baseline clinical and laboratory characteristics

A total of 195 patients were included in the TiN cohort. For this study, patients with a normal ISTH-BAT score (n=33) and patients with an acquired platelet function disorder (n=4) were excluded, resulting in a final study population of n=158 (figure 1). Patient characteristics of the 158 included patients are shown in table 1. The majority of the patients was female (128/158, 81%), the median age was 43 [31-54] years in women and 45 [31-61] years in men. The median bleeding score was 11 [9-14] in females and 10 [7-13] in men. The mean leukocyte and platelet count were within reference ranges, 34 patients had a platelet count <150 10^9/L and 7/34 had a platelet count <100 10^9/L. A platelet count of 100 - 150 10^9/L was not considered to be explanatory for an increased bleeding tendency.

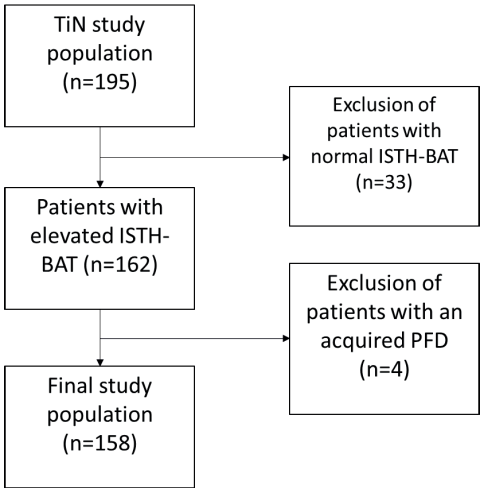

Figure 1: Flow diagram of study population. Patients with normal ISTH-BAT scores and acquired PFD were excluded from the TiN study population, resulting in a final study population of n=158.

TiN, thrombocytopathy in the Netherlands; ISTH-BAT, international society of Thrombosis and Hemostasis bleeding assessment tool; PFD, platelet function disorder.

Results of different platelet (function) assays in TiN patients

Among the 158 patients included, 34 had a platelet count below 150 × 10^9/L. Of these, 19 presented with a platelet count below 100 × 10^9/L. Seven of these 19 patients were diagnosed with isolated thrombocytopenia, as the low platelet count was their only abnormal test result. The remaining 12 patients showed additional abnormalities and were therefore diagnosed with a platelet function disorder. Among the 15 patients with platelet counts between 100 and 150 × 10^9/L, six were classified as having BDUC, since no other platelet function abnormalities were identified.

Results of the different platelet function assays in the TiN patients are displayed in table 2. For each test, the reference values, median value and interquartile ranges and the number of observed abnormalities in 158 included patients are shown. LTA could not be performed in 12 patients due to insufficient platelet count in PRP (<75 10^9/L). 66/146 patients (45%) had abnormal LTA results, with abnormal responses towards two or more agonists in 54 patients. ADP platelet content levels were measured in 145/158 patients. Reasons for missing ADP platelet content levels were low platelet count (8/13), shortage of material or technical difficulties (5/13). In 14/145 (10%) patients, platelet ADP content was below the reference range, indicative of delta storage pool disease. Of these 14 patients, 8 patients also had abnormal LTA results.

Table 1: Baseline clinical and laboratory characteristics in patients suspected for a platelet function disorder (n=158).

Clinical characteristics		**n=158**
Sex, female, n (%)		128 (81)
Age (years)		
Women, median [IQR]		43 [31-54]
Men, median [IQR]		45 [31-61]
Bleeding score		
Women median [IQR]		11 [9-14]
Men, median [IQR]		10 [7-13]
Laboratory characteristics	**Reference values**	**n=158**
Hb (mmol/L)	7,5-10	
Women, mean ± SD	8,5-11	8,4 [7,8-8,9]
Men, mean ± SD		9,1 [8,8-9,9]
Leukocyte count (10^9/L), mean ± SD	4-10	6 [5-8]
Platelet count (10^9/L), mean ± SD	150-450	[165-274]

SD, standard deviation; Hb, hemoglobin

Flow cytometry-based receptor expression was measured in all 158 patients. Abnormal receptor expression was found in one or more receptors in 18/158 patients (11%). The GP1b receptor expression was abnormal in 2/18 patients, the CD49b receptor expression was abnormal in 4/18 patients and the CD41a receptor expression was abnormal in 12/18 patients. No abnormal results were found for the GPVI receptor. In 16/18 patients, LTA and/or platelet ADP content were abnormal as well. In the remaining 2/18 patients, LTA and platelet ADP content were not determined due to insufficient platelet count in PRP.

PACT showed abnormal results for one or more agonists in 58/158 patients (37%). 47/58 patients (81%) also had abnormal LTA, platelet ADP content and/or flow cytometry receptor expression results. In 40/58 patients (25%), 2 or more agonists were abnormal. In 28/58 (48%) of the patients with abnormal PACT, P-selectin expression was decreased in response to one or more agonists, suggesting a defect in the alpha granules of the platelets, which can't be tested with the other secretion assays.

Based on the results of these platelet function assays, 91 patients were diagnosed with an IPD. In 67 patients with an increased bleeding tendency, no explanation for their bleeding symptoms was found and these patients were classified as having a bleeding disorder of unknown cause (BDUC). In figure 2, the combinations of abnormal platelet function

test results are shown. This figure shows that LTA yielded the most abnormal results, followed by PACT, flow cytometry receptor expression and platelet ADP content. A total of 13 different combinations of abnormal test results were observed. The combination of an abnormal LTA and abnormal PACT was most common, followed by LTA alone and the combination of abnormal LTA, PACT and flow cytometry receptor expression.

Table 2: Results of the different platelet function tests in the TiN patients (n=158).

Assay	Reference values	Median [IQR] TiN	Number of abnormal observations (n=)
Light transmission aggregometry (LTA)			**Total: 66/146**
ADP 5.0 µM (MA %)	>73	78 [62-85]	53
Arachidonic acid 1.5 mM (MA %)	>60	74 [50-79]	39
Collagen 4.0 µg/mL^{-1} (MA %)	>78	86 [80-89]	23
Ristocetin 1.0 mg/mL^{-1} (MA %)	>75	84 [80-87]	24
Platelet ADP concentration			**Total: 14/145**
Platelet ADP content (µmol/10^{11} platelets)	1.4-3.8	2.5 [2.0-2.9]	14
Flow cytometry based receptor expression			**Total: 18/158**
Receptor_GP1b (MFI)	656 – 2461	2748 [2401-3380]	2
Receptor_CD49b (MFI)	304 – 899	558 [458-670]	4
Receptor_CD41a (MFI)	5650 – 30491	17373 [15090-22110]	12
Receptor_GPVI (MFI)	839 – 3210	2812 [2277-3222]	0
Flow cytometric Platelet Activation Test (PACT)			**Total: 58/158**
VWF_binding (MFI)	151-3253	271 [211-352]	7
Fibrinogen_PAR1 (MFI)	917 – 2665	2042 [1554-2486]	8
Fibrinogen_PAR4 (MFI)	1157 – 2611	2045 [1573-2441]	19
Fibrinogen_ADP (MFI)	467 – 2132	793 [587-1050]	21
Fibrinogen_CRP (MFI)	578 – 4188	1563 [1081-2082]	8
Fibrinogen_U46619 (MFI)	7434 – 2458	1137 [815-1528]	31
P-selectin_PAR1 (MFI)	15901 – 3800	3883 [3289-4187]	0
P-selectin_PAR4 (MFI)	2030 – 3964	3830 [3274-4303]	6
P-selectin_ADP (MFI)	529 – 2831	1078 [808-1409]	9
P-selectin_CRP (MFI)	1075 – 5590	3289 [2353-4131]	13
P-selectin_U46619 (MFI)	989 – 3632	1937 [1322-2808]	16

Reference ranges, medians [IQR] and the number of abnormal results observed in the TiN patients (n=158). ADP, adenosinediphosphate; MA maximal amplitude; GP, glycoprotein; CD, cluster of differentiation; MFI, mean fluorescence intensity; PAR, protease activated receptor; ADP, adenosine diphosphate; CRP, C reactive protein; U46619, thromboxane A2 analog.

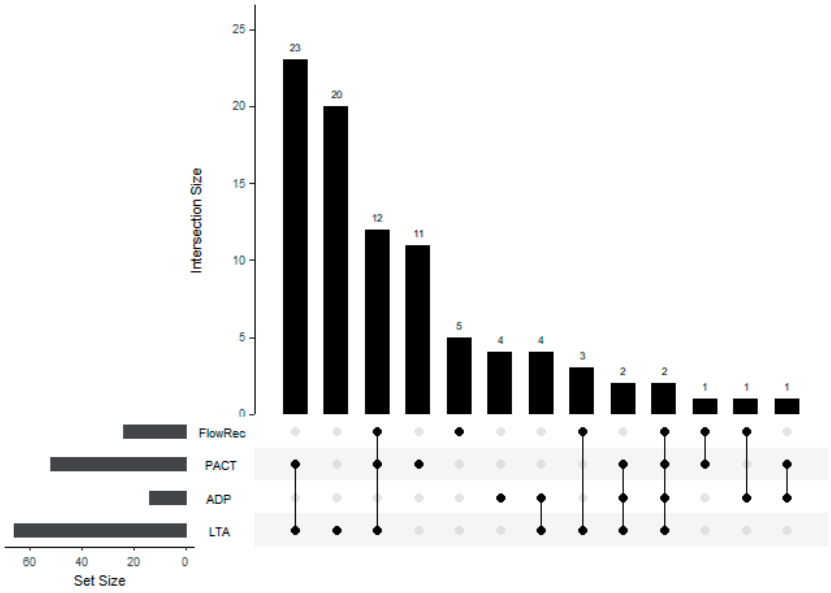

Figure 2: Plot of combinations of abnormal test results in 158 included patients. Horizontal bars on the left side of the plot show the amount of abnormal test results for each platelet function test. Vertical bars show the number of patients that had that specific combination of abnormal test results, indicated by the dots and connected lines.

ADP, adenosine diphosphate; FlowRec, flow cytometry based receptor expression; PACT, flow cytometry based platelet activation test; LTA, light transmission aggregometry.

Agreement between diagnostic tests and their diagnostic yield

The diagnostic yield for all platelet function tests performed in this study was assessed based on current clinical practice in our center with LTA as the gold standard (figure 3). Figure 3 displays the total diagnostic yield of each test and additional diagnostic yield of platelet ADP content, flow cytometry receptor expression and PACT on top of LTA. LTA had a diagnostic yield of 45% and platelet ADP content had an additional diagnostic yield of 8%, flow cytometry based receptor expression had an additional diagnostic yield of 3% and PACT identified an additional 19% of patients with an IPD. Although there was considerable overlap between tests, agreement between tests was low: only LTA and PACT showed moderate agreement ($\kappa = 0.41$) (figure 4).

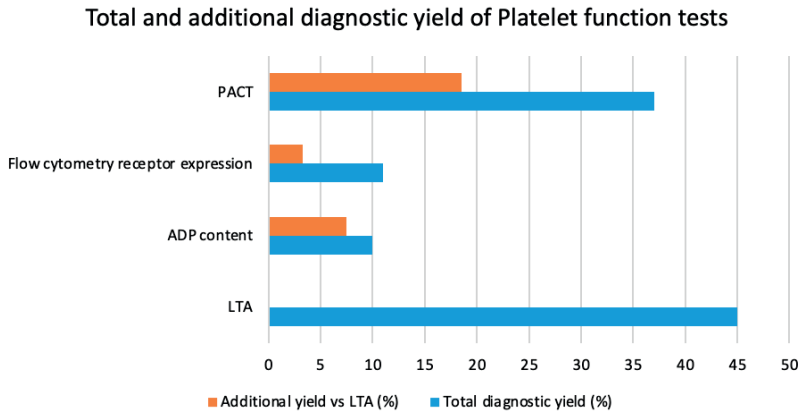

Figure 3: Diagnostic yield and additional diagnostic yield of platelet function assays. The blue bar graph illustrates the total diagnostic yield of each platelet function test and the additional diagnostic yield (orange) relative to light transmission aggregometry (LTA), which served as the reference assay.

ADP, adenosine diphosphate; LTA, light transmission aggregometry; FLowRec, flow cytometry based receptor expression; PACT, flow cytometry based platelet activation test.

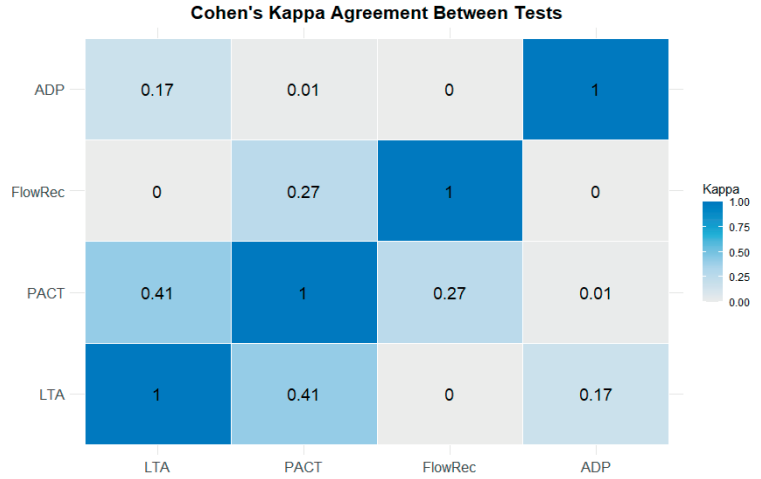

Figure 4: Heatmap of pairwise agreement between platelet function tests calculated using Cohen's kappa coefficient (κ). Higher values indicate stronger agreement with κ=1 representing perfect agreement and κ=0 indicating agreement no better than chance. Only pairwise complete observations were used.

ADP, adenosine diphosphate; LTA, light transmission aggregometry; FLowRec, flow cytometry based receptor expression; PACT, flow cytometry based platelet activation test.

DISCUSSION

This study described the diagnostic value of light transmission aggregometry (LTA), platelet ADP content, flow cytometry based receptor expression and PACT in patients with an increased bleeding tendency and a suspected inherited platelet defect. Our results showed that LTA had the highest diagnostic yield, identifying abnormalities in 45% of patients. The platelet ADP content assay identified an additional 8% of patients with a dense granule deficiency. Flow cytometry based receptor expression yielded another 3% of IPD diagnoses and PACT 19%. In total, 91 of 158 patients (58%) were diagnosed with an IPD using the combination of these assays, while the remaining 67 patients (42%) showed no abnormalities and were classified as having a bleeding disorder of unknown cause (BDUC).

Overall, these findings indicate that LTA remains the platelet function test with the highest diagnostic yield in this patient population. The other, more specialized, platelet function tests contribute to a higher diagnostic yield in patients with no other hemostasis abnormalities and a high suspicion for a primary hemostasis defect. The LTA is well known to be the gold standard platelet function test that was developed in 1962 (16-18). LTA is a time consuming assay and it requires well-trained laboratory technicians because of the many pre-analytical and analytical variables that can influence LTA results (18). The technique poses many challenges including the high volumes of PRP needed and the time-consuming aspect of the test. LTA also has some limitations, as it is unable to measure platelet aggregation in samples with low platelet count and it is limited in identifying mild IPD and platelet secretion defects (10, 12). Despite these limitations, our study supports the continued use of LTA as the cornerstone of diagnostic workup in patients with suspected platelet function disorders.

The PACT had the highest additional diagnostic yield on top of the LTA. Previous studies have shown that the PACT provides added diagnostic value on top of the LTA (19). The added diagnostic value could be explained by the use of different agonists in the PACT and LTA, causing both tests to identify different subpopulations (19). The major advantage of this flow cytometry based test is the small sample value of only 100 µL. This provides opportunities for platelet function testing in children. Moreover, the PACT can be performed in patients with low platelet count (20, 21). On the other hand, a large overlap was seen between the LTA and the PACT, which could argue for the use of one of both assays, as they probably identify mostly the same patients.

In contrast to the PACT, the flow cytometry-based receptor expression assay did not substantially increase the overall diagnostic yield in this cohort. Rather, its main value lies in confirming and further specifying diagnoses in patients with already abnormal LTA

results or in those with known macrothrombocytopenia or suspected platelet glycoprotein defects. This assay, recommended by the ISTH SSC guidelines as part of the diagnostic workup for inherited platelet disorders, is particularly useful for evaluating the expression of platelet glycoproteins such as GPIIb/IIIa, GPIb/IX, GPIa/IIa, and GPVI (13). In the present study, flow cytometry receptor expression measurements included GPIa, GPIIb/IIIa, GPIb, and GPVI. Although the assay has limited additional diagnostic value as a functional test, it remains highly valuable for diagnostic confirmation and subclassification of platelet disorders. Moreover, because only a small sample volume is required, flow cytometry-based receptor expression testing represents the preferred platelet function assay in neonates and pediatric patients, where sample availability is often restricted.

Because LTA, PACT and flow cytometry-based receptor expression are not able to detect platelet secretion defects, a secretion assay is indicated. In this study, the platelet ADP content assay identified 14 patients with abnormal secretion, including 8 who also had abnormal LTA results. Although the additional diagnostic yield of 8% of this test might seem rather limited, it is important to consider this assay (or another platelet secretion assay) in patients with a high suspicion for an IPD with normal LTA results, but also in patients with abnormal LTA results as aggregation and secretion defects can co-exist (22).

Our findings highlight the value of a structured, comprehensive approach to the diagnostic workup of suspected IPDs. Given the highest diagnostic yield of LTA, it should remain the first-line platelet function test in patients with a bleeding tendency and a suspected IPD. However, the addition of specialized assays, such as secretion assays, is recommended as it is complementary to the LTA and delivers unique information. The PACT also showed an additional diagnostic value, and could be especially relevant in patients with a strong clinical suspicion of a primary hemostatic defect but normal LTA results and in patients with thrombocytopenia in whom LTA is not possible.

The subgroup of patients classified as BDUC remains challenging. While no abnormalities were found using the available platelet function assays, subtle functional or regulatory defects may still be present. Future studies could explore the role of whole blood microfluidic assays or platelet proteomics in uncovering the underlying bleeding mechanism.

Our study has several limitations that should be considered when interpreting the results. First, it was conducted in a single tertiary care center with access to in-house developed platelet function assays, such as the PACT, which may limit the generalizability of the findings to other settings. Second, the study population was heterogeneous, reflecting the clinical diversity of patients referred for evaluation of a suspected IPD, which may also have introduced variability that complicates the interpretation of assay performance

and abnormality patterns. Furthermore, not all tests could be performed in all patients due to factors such as thrombocytopenia, limited sample availability, or technical issues. This led to unequal group sizes and may have affected the ability to directly compare the diagnostic yield of the different assays.

This study provides a conceptual framework for future research in platelet function disorders. Advanced diagnostic approaches, including flow-based assays, expanded receptor panels, secretion assays, platelet proteomics, and targeted genetic testing, may help uncover currently undetectable defects and further stratify patients. Integration of these techniques into multidimensional diagnostic workflows will enhance mechanistic understanding and may ultimately inform individualized management strategies for both IPD and BDUC patients.

In conclusion, LTA remains the cornerstone of IPD diagnostics, while PACT and platelet ADP content assays provide complementary information that increases overall diagnostic yield. Flow cytometry-based receptor expression confirms suspected defects or enables testing in contexts where LTA is not feasible. Patients without detectable abnormalities remain challenging, highlighting the need for future studies using advanced functional and molecular assays.

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CHAPTER 8

MICROFLUIDIC TESTING IN PATIENTS WITH BLEEDING DISORDER OF UNKNOWN CAUSE (BDUC)

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Submitted

ABSTRACT

Background: In 40–70% of patients referred to a hematologist for increased bleeding tendency, no diagnosis is established despite extensive laboratory testing. These patients are classified as having a bleeding disorder of unknown cause (BDUC), often presenting with a bleeding phenotype similar to known mild bleeding disorders, including platelet function defects.

Aim: This study aimed to assess whether a microfluidic assay can detect shear-dependent hemostatic defects in BDUC patients.

Methods: BDUC patients with elevated ISTH-BAT scores (≥6 for women and ≥4 for men) and normal coagulation factor levels, von Willebrand factor levels, platelet aggregation by light transmission aggregometry (LTA), and ATP release were selected from the Predictors of Bleeding Evaluation in Adult Hematologic Patients (ProBe-AHP) cohort. Shear-dependent platelet adhesion, activation, and aggregation were evaluated using microfluidic analysis of collagen- or collagen plus tissue factor-induced whole blood thrombus formation at 37 °C.

Results: Abnormal shear-dependent platelet function was observed in 25 of 47 (53%) BDUC patients, with a mean ISTH-BAT score of 10. Reference ranges were based on measurements in 49 healthy controls. Affected patients exhibited reduced platelet adhesion, integrin activation, phosphatidylserine exposure, and/or P-selectin expression. Additionally, thrombus morphology, contraction, and multilayer formation were impaired in 5 patients. Fibrin formation on collagen with tissue factor did not differ significantly from controls.

Conclusion: Microfluidic analysis of whole blood thrombus formation under shear identifies abnormalities in over half of BDUC patients, providing more detailed insights into flow-dependent platelet abnormalities and supporting a flow-related platelet defect diagnosis.

INTRODUCTION

Patients with an increased bleeding tendency are often referred to a hemostasis expert for medical evaluation. About 40-70% of these patients remain undiagnosed after extensive laboratory testing [1]. This group of patients is classified as having a 'Bleeding Disorder of Unknown Cause' (BDUC) [2]. Common bleeding symptoms seen in BDUC patients are similar to those of patients with mild bleeding disorders (MBDs) and include epistaxis, heavy menstrual bleeding (HMB), bruising and bleeding during or after surgical and/or dental interventions. The majority of BDUC patients is female, probably due to women-specific challenges such as HMB or postpartum hemorrhage (PPH) [1, 3]. BDUC patients report an impaired health-related quality of life [4], emphasizing the burden of the disease and the urgent need for standardized guidelines and management strategies, particularly given the limited understanding of the underlying bleeding mechanisms [4].

A minimum laboratory workup for BDUC should include a complete blood count, prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time, fibrinogen, von Willebrand Factor (vWF) antigen and activity, coagulation factors (F) VIII, FIX, and FXI, as well as platelet function testing using light transmission aggregometry (LTA) [5]. Moreover, the use of a platelet secretion assay is advised, if available. In patients with BDUC, the use of other specialized hemostasis tests to exclude rare causes of bleeding could be considered, including other platelet assays such as the platelet function analyzer 200 (PFA-200) and flow cytometry-based platelet assays, but also fibrinolysis assays and tests for rare coagulation factor deficiencies, as the currently available standard hemostasis assays fail to explain the underlying cause of bleeding [6-8]. The majority of these laboratory tests, except for the PFA-200, uses plasma, is conducted under static conditions and does not incorporate physiological blood flow. Therefore, more diagnostic research is needed to develop novel laboratory tests, better mimicking the in vivo situation, able to identify pathophysiological bleeding mechanisms in BDUC patients [6].

Multiparameter microfluidic assays, such as the Maastricht flow chamber, provide another approach to investigate whole-blood thrombus formation under near-physiological conditions [9-11]. The Maastricht flow chamber, developed by the Biochemistry department at Maastricht University, enables the investigation of hemostatic processes under controlled conditions. It uses whole blood and can simulate physiological shear stress, blood flow and body temperature (37°C) [12]. Multiparameter assessment of thrombus formation provides detailed insights into platelet adhesion, activation, aggregation and fibrin formation under flow conditions. The Maastricht flow chamber has previously been able to successfully detect abnormalities in patients with known platelet function deficiencies, other known bleeding disorders and immune deficiencies [9, 11, 13]. The use of thrombogenic surfaces coated with microspots of collagen type I and type

III makes it possible to study thrombus formation under conditions resembling vascular injury in vivo [14].

Among the components of the damaged vessel wall, fibrillary collagen types I and III are recognized as key platelet-activating elements that regulate thrombus formation [15, 16]. At arterial wall shear rates, the initial phase of platelet rolling is primarily driven by the interaction between the platelet GPIb receptor and vWF, which is bound to the exposed collagen with a higher affinity for collagen type III [17]. This initial contact is reinforced by platelet activation through collagen receptors such as glycoprotein VI (GPVI) and integrin α2β1, alongside integrin αIIbβ3, the receptor for fibrinogen [18]. Platelet activation via collagen leads to dense and α-granules secretion (as indicated by P-selectin expression). The secreted mediators serve to recruit and activate circulating platelets. Eventually, these mediators and GPVI modulate the affinity of integrins α2β1 and αIIbβ3, involved in the stabilization of the thrombus. Furthermore, GPVI signaling enhances the procoagulant activity through exposure of phosphatidylserine (PS) exposure on the platelet surface [19]. PS exposure provides a procoagulant surface on the platelets where the coagulation cascade can take place, resulting in thrombin generation and eventually fibrin formation [20]. Different aspects of these processes can be measured with the Maastricht flow chamber, including fibrinogen binding, P-selectin expression, PS-exposure and fibrin formation, using fluorescent labels.

A number of BDUC patients report a prolonged platelet function PFA-200 closure time as the only aberrant test result [9, 21]. The PFA-200 test is used as a screening test for primary hemostasis under conditions of high wall shear rates. While the test is able to detect severe forms of VWD and PFD, its limited sensitivity to mild cases raises questions about its diagnostic value for patients with bleeding tendencies [9, 10]. However, it is the only clinical test using flow conditions so far. Unlike the PFA-200, which operates at higher shear rates, the Maastricht flow chamber mimics physiological wall shear stress more closely [9].

Our previous study examined whether the Maastricht flow chamber was able to identify a primary hemostasis defect in a small group of patients with abnormal PFA-200 results, but normal LTA results and normal vWF activity and antigen levels. By applying the Maastricht flow chamber, we found a shear-dependent platelet function defect in a part of these patients, which was not detectable by standard laboratory tests [9]. In the current study, a larger patient population was included, consisting of BDUC patients with or without prolonged PFA-200 results, in whom thrombus formation, and fibrin formation are assessed. The aim of this study was to investigate whether a multiparameter flow model, such as the Maastricht flow chamber, can identify shear-dependent hemostasis defects in BDUC patients.

MATERIALS & METHODS

Study population

Clinical and laboratory data from the Predictors of Bleeding Evaluation in Adult Hematologic Patients with Bleeding Tendencies (ProBe-AHP) cohort (METC 14-4-036, NL9643) was used for this study. The ProBe-AHP study is an observational study in which adult patients who were referred to a hematologist for bleeding evaluation are included. The study has been described in more detail previously [22, 23]. In all patients the International Society of Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH BAT) [24] and standard coagulation tests were performed during a first visit. An overview of the coagulation tests used in these patients can be found in Supplementary Table 1. The diagnostic criteria for BDUC are an increased bleeding tendency, based on an elevated ISTH BAT score (≥6 for women and ≥4 for men), without abnormal laboratory findings except for a prolonged PFA-200 as the sole abnormality. For this study, clinical and laboratory data collected from July 2021 until May 2025 were used. BDUC patients were invited for additional investigations using the Maastricht flow chamber. The selection of BDUC patients was based on the severity of their bleeding phenotype (measured with the ISTH-BAT score). All participating patients gave written informed consent according to the Helsinki declaration. Patients using anti-platelet agents or anticoagulant therapy were excluded. The use of selective serotonin reuptake inhibitors (SSRI's), nonsteroidal anti-inflammatory drugs (NSAIDs) and any other medication and/or herbal preparations/ supplements to have a known influence on the hemostatic process was documented. Patients were either asked to discontinue the use of these products at least two weeks in advance. If discontinuation was impossible, patients were excluded from the study.

Healthy volunteers

Data from forty-nine healthy volunteers were used as an unmatched reference group for the Maastricht Flow chamber assay. Healthy volunteers were recruited amongst employees and students from the central diagnostic laboratory of the Maastricht University Medical Centre+ (MUMC+) (METC152015, NL52640.068.15). All participating healthy volunteers gave written informed consent according to the Helsinki declaration. The use of any medication and/or herbal preparations/supplements to have a known influence on the hemostatic process was excluded.

Blood collection

Blood was drawn from the antecubital vein by venipuncture using a 21G needle. EDTA blood (BD Vacutainer; Becton Dickinson) was used for complete blood counts. Citrated blood (Vacuette, Greiner bio-one) was used for the Maastricht Flow chamber assay and the PFA-200. Immediately after blood withdrawal, 100 µL of thermostable inhibitor of contact activation (TICA) (50 µg/mL) was added to one of the 9 mL citrated tubes, before

mixing it. The remainder of the citrated blood was centrifuged at 2490 g for five minutes at 18 ℃ (Universal 320 R, Hettich Zentrifugen) and a second time at 10000 g for ten minutes at 18℃ to obtain platelet poor plasma (PPP). PPP was used for routine coagulation tests (PT, aPTT and fibrinogen). Remaining PPP was frozen at -80 ℃. Frozen PPP was used for determination of coagulation factor levels and von Willebrand factor levels.

Laboratory measurements

Hemoglobin (Hb), platelet and white blood cell (WBC) counts were measured on a Sysmex XN-9000 analyzer. Routine hemostasis tests, consisting of PT (Innovin; Siemens), aPTT (ActinFSL; Siemens), fibrinogen activity (Clauss Method, Thrombine reagent; Siemens) and FXIII activity (Berichrom FXIII, Siemens), were measured on a Sysmex CS-2500 coagulation analyzer. Coagulation factor levels FVIII, FII (neoplastine R), FV, FVII, FVIII, FIX, FX (neoplastine R) and FXI were measured with a clotting assay using factor depleted plasma on a Sta-R apparatus (Stago Diagnostics, Paris, France). VWF antigen and activity levels were measured on a Sysmex CS2100i analyzer (VWF antigen Reagens and VWF Activity Reagens, Siemens). The platelet function analyzer 200 (PFA-200; Innovance PFA-200 System, Siemens, Marburg, Germany) was used. Closure times (CT) were measured with collagen and epinephrine (C-EPI) or collagen and ADP (C-ADP) kits.

Maastricht flow chamber

The Maastricht flow chamber assay was used for thrombus formation evaluation, as was previously described in detail [9, 10, 25]. The experiments were performed at a physiological temperature of 37°C [11]. Brightfield and tri-color fluorescence images were taken with an EVOS-FL microscope (Life Technologies). Two different protocols were used. The first protocol used two coated micro spots (collagen I and collagen III). Two runs were performed. The first run had a perfusion time of 7 minutes. DOIC6 fluorescent label was added and right before the start of the perfusion, whole blood was recalcified. Platelet adhesion, aggregation and activation were assessed on the collagen I micro spot using different parameters, including (i) platelet surface area coverage measured in brightfield (BF platelet SAC), representing regions of all adhered platelet and thrombus structure, (ii) platelet surface area coverage measured with DIOC6 fluorescent label (DIOC6 platelet SAC), representing regions of platelet deposition and mono- or multi-layered thrombi, (iii) thrombus morphology score (0-6), (iv) thrombus contraction score (0-4) and (v) thrombus multilayer score (0-4). The second run had a perfusion time of 10 minutes. Different fluorescent labels (FITC-α-fibrinogen mAb, Annexin A5, Alexa Fluor 568 and AF647-α-CD62P mAb (P-selectin)) were added to the whole blood and it was recalcified right before the start. Platelet surface area coverage was measured for (vii) $\alpha_{IIb}\beta_3$ activation, (viii) phosphatidylserine (PS) exposure and (ix) P-selectin expression. Platelet adhesion and aggregation and the fluorescence parameters were assessed at a collagen I surface and the blood was pushed at a wall-shear rate of 1000 s^{-1}. VWF-dependent platelet adhesion

was assessed on a collagen III surface under the same circumstances. Both runs were performed in duplo for each patient and healthy control. An overview of the different parameters can be found in Table 1.

The second protocol used three coated micro spots (collagen I + tissue factor (TF), collagen I and collagen III). The fluorescent labels DIOC6, fibrinogen label (AF647) and Annexin A5, Alexa Fluor 568 were added to the whole blood and it was also recalcified before the start of the perfusion, which lasted for 10 minutes. Blood was pulled at a wall-shear rate of 1000 s^{-1}. This protocol was primarily used to assess fibrin formation (x) (%SAC) on the collagen I + TF micro spot (Table 1). This run was also measured in duplo. The development of the second protocol is co-funded by the PPP Allowance made available by Health-Holland, Top Sector Life Sciences & Health to stimulate public-private partnerships. ImageJ was used for the analysis of the microscopic images. Morphology, contraction and multilayer scores were assessed visually compared to scored reference brightfield images.

Table 1: Overview of microfluidic parameters and their mechanisms.

Parameters	Range	Micro spot surface	Running time
(i) Brightfield platelet surface area coverage (%SAC)	0-100	Collagen type I	7 minutes
(ii) DIOC6 platelet surface area coverage (%SAC)	0-100	Collagen type I	7 minutes
(iii) Thrombus morphology score	0-6 (0= no adhesion; 6=large aggregates)	Collagen type I	7 minutes
(iv) Thrombus contraction score	0-4 (0=no thrombus contraction; 4=fully contracted thrombus)	Collagen type I	7 minutes
(v) Thrombus multilayer score	0-4 (0= platelet monolayer; 4= multilayered thrombus)	Collagen type I	7 minutes
(vi) Brightfield surface area coverage (%SAC)	0-100	Collagen type I and collagen type III	10 minutes
(vii) $\alpha_{IIb}\beta_3$ activation (%SAC)	0-100	Collagen type I	10 minutes
(viii) PS-exposure (%SAC)	0-100	Collagen type I	10 minutes
(ix) P-selectin expression (%SAC)	0-100	Collagen type I	10 minutes
(x) Fibrin formation (%SAC)	0-100	Collagen type I + TF	10 minutes

SAC, surface area coverage; PS, phosphatidylserine, TF, tissue factor.

Statistical analysis

Continuous variables were presented as either mean ± standard deviation for normally distributed data or median ± interquartile ranges for non-parametrical data. Categorical variables were shown as counts and percentages. In case of normal distribution, differences between two groups were calculated using an unpaired t-test. For not normally distributed data, the Mann-Whitney U test was used and for categorical data, the Fisher's exact test was used. Statistical significance is assumed at a p-value <0.05. For statistical analysis, Graph Pad Prism (version 5.04) and IBM SPSS statistics version 28 were used.

RESULTS

Reference values in healthy volunteers

Reference values were calculated based on Maastricht flow chamber results from 49 healthy volunteers with a mean age of 35 years and 37% (18/49) males. Supplementary Table 2 shows the cut-off values to discriminate normal from abnormal results, based on the 2.5th and 97.5th percentiles of the results in 49 healthy controls.

Baseline characteristics

A total of 47 BDUC-patients from the ProBe-AHP study were analyzed by the Maastricht Flow chamber. Baseline characteristics and laboratory measurements are shown in Table 2. Only one male patient was included. The median [IQR] age was 37 [29-52] years. The mean ISTH-BAT score in was 10 ± 3 in women and the one male patient had an ISTH-BAT of 6. As expected in BDUC patients, all baseline laboratory measurements means and medians were within reference ranges. Looking at minimal and maximal ranges, in some individual patients, isolated abnormalities in a single coagulation test were observed, but these were considered insufficient to explain their overall bleeding phenotype.

BDUC patients vs. reference ranges

Results from individual BDUC patients were plotted against the reference ranges established by the results of the healthy volunteers (n=49). 10 BDUC patients showed platelet SAC below the reference ranges, 11 patients demonstrated platelet deposition (DIOC6 SAC) below reference ranges, five patients had decreased morphology scores and four patients had both decreased contraction and multilayer scores (Figure 1A).

Platelet SAC after 10 minutes on the collagen I spot was below reference ranges in five BDUC patients. Looking at VWF-dependent platelet aggregation on the collagen type lll micro spot, eight BDUC patients were below reference ranges in platelet SAC. Regarding the fluorescent labels on the collagen I spot, $\alpha_{IIb}\beta_3$ activation was below reference ranges in three patients, PS exposure was below reference ranges in five patients and P-selectin

Table 2: Baseline characteristics in BDUC patients (n=47).

Patient characteristics		**BDUC (n=47)**
		Median [IQR], Mean ± SD, n (%)
	Normal range	
Age in years		37 [29-52]
Female, n (%)		46 (98)
ISTH-BAT score	0-5 in women	10 ± 3
	0-3 in men	6
Laboratory values	**Normal range**	**Mean ± SD, median [IQR], (min - max range)**
Hemoglobin (g/dL) - male	13.2-17.7	14.8
Hemoglobin (g/dL) - female	11.8-15.6	13.9 [13.5-14.3], (10.2-18.0)
Leukocytes ($x10^9$/L)	3.5-11	6.0 [5.1-7.6], (3.6-12.8)
Platelets ($x10^9$/L)	130-350	282 [241-322], (82-680)
aPTT (s)	23-32	28 [27-30], (25-34)
PT (s)	9.9-12.4	10.7 [10.4-10.9], (9.8-12.9)
Fibrinogen (g/dl)	1.7-4.0	3.0 [2.7-3.5], (1.6-6.4)
PFA EPI (s)	84-160	129 [108-155], (76-268)
PFA ADP (s)	71-118	100 [85-116], (69-191)
VWF antigen (%)	50-150	99 [72-128], (55-326)
VWF activity (%)	50-150	101 [70-135], (54-333)
FII (%)	60-140	115 ± 18, (79-154)
FV (%)	60-140	106 ± 27, (61-162)
FVII (%)	60-140	105 ± 27, (14-153)
FVIII (%)	50-200	120 [95-164], (56-280)
FIX (%)	60-140	111 ± 20, (72-156)
FX (%)	60-140	109 ± 20, (65-158)
FXI (%)	60-140	122 ± 23, (70-168)
FXIII (%)	70-140	124 ± 30, (49-184)

ISTH-BAT, International society on thrombosis and hemostasis bleeding assessment tool; aPTT, activated partial thromboplastin time; PT, prothrombin time; PFA, platelet function analyzer; EPI, epinephrine; ADP, adenosine diphosphate; VWF, von Willebrand factor; F, coagulation factor.

expression was below reference ranges in eight patients (figure 1B). In figure 2, microscopic pictures of a healthy control and one of the BDUC patients are shown for illustration.

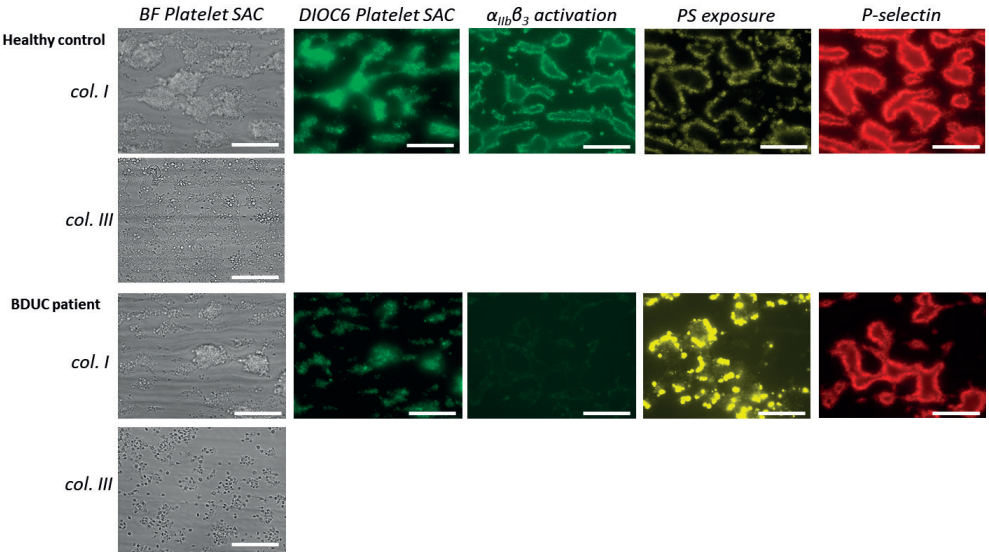

Figure 2: Microscopic imaging of platelet thrombus formation on collagen type l and lll in a healthy control and a BDUC patient.

Images are collected from one healthy volunteer and one BDUC patient (patient 2 from table 5) to illustrate the different parameters measured on the collagen type I and III microspots. SAC, surface area coverage; col I, collagen type 1; col III, collagen type 3; PS, phosphatidylserine. Scale bar = 40 µm.

Fibrin formation in BDUC vs healthy volunteers

Fibrin formation on the TF/collagen I spot was compared between 33 BDUC patients and 27 healthy volunteers. No significant differences were observed at any of the measured time points between BDUC patients (n=33) and healthy volunteers (n=27) (Figure3A). Figure 3B shows microscopic images of fibrin formation after 10 minutes in a healthy control and a BDUC patient.

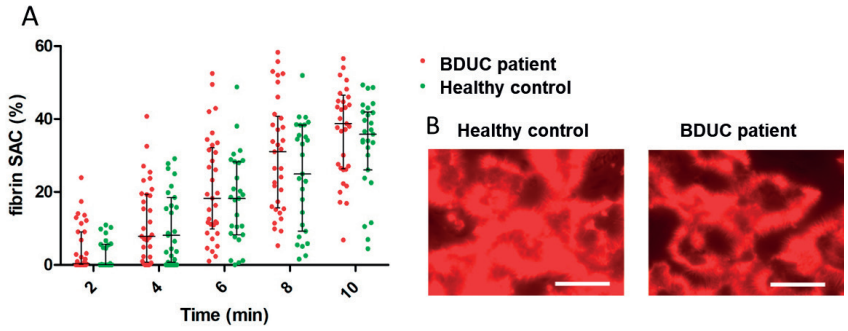

Figure 2: Microscopic imaging of platelet thrombus formation on collagen type l and lll in a healthy control and a BDUC patient.

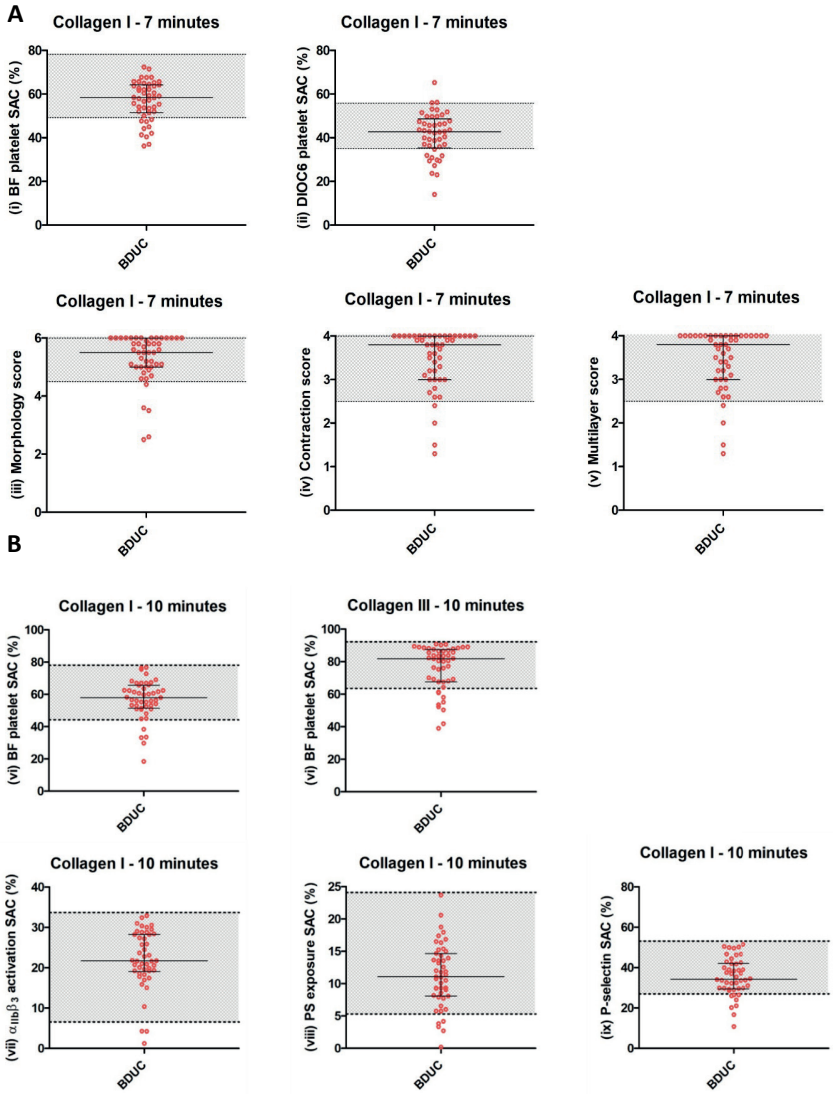

Figure 1: Abnormal platelet adhesion and aggregation results in BDUC patients (n=47) (A) and (B) abnormal VWF-dependent platelet adhesion and fluorescent label results in BDUC patients (n=47).

Scatterplots show the median with interquartile ranges of platelet adhesion and aggregation parameters at 7 minutes in panel A: the bright field platelet surface area coverage (i), DIOC6 platelet surface area coverage (ii), the morphology score (iii), the contration score (iv) and the multilayer score (v). In panel B, medians with interquartile ranges are shown for bright field platelet SAC at collagen 1 surface at 10 minutes (vi), bright field platelet SAC at collagen 3 surface at 10 minutes (vi), αIIbβ3 activation (vii), PS exposure (viii), and P-selectin expression (ix) measured at the collagen type l spot. Reference ranges are indicated with grey areas. SAC, surface area coverage; PS, phosphatidylserine.

The graph shows the fibrin surface area coverage measured at 2,4,6,8 and 10 minutes of running time. A comparison was made between BDUC patients and healthy volunteers. Mann Whitney U tests were used to test for significance (A). Images are collected from one healthy volunteer and one BDUC patient to illustrate the fibrin formation at 10 minutes on the collagen type I + TF microspot (B). SAC, surface area coverage.

BDUC patients with abnormal flow chamber results

	Adhesion and aggregation		Aggregation & activation			Adhesion (VWF-dependent on collagen 3)		Activation and aggregation	Activation	Activation & Secretion	
Patient Nr.	**BF platelet SAC**	**DIOC6 Platelet SAC**	**Morphology score**	**Contraction score**	**Multilayer score**	**BF platelet SAC (col 1)**	**BF platelet SAC (col3)**	**$\alpha_{IIb}\beta_3$ (col 1)**	**PS exp (col 1)**	**P-selectin (col 1)**	**Nr. Of abnormalities**
1(PFA)	X	X	X	X	X	X	X	X		X	9
2 (PFA)	X	X				X	X		X	X	6
3	X	X				X	X				4
4		X					X		X		3
5 (PFA)	X	X					X				3
6	X	X					X				3
7						X			X	X	3
8 (PFA)			X				X			X	3
9 (PFA)	X	X					X				3
10 (PFA)			X	X	X						3
11			X	X	X						3
12			X	X	X						3
13	X					X				X	3
14	X	X						X			3
15	X	X									2
16										X	1
17		X									1
18 (PFA)										X	1
19								X			1
20							X				1
21		X									1
22 (PFA)										X	1
23	X										1
24 (PFA)									X		1
25									X		1

A total of 25/47 (53%) BDUC patients showed one or more abnormal parameters in the Maastricht flow chamber. The total number of abnormal parameters found for each BDUC patient varied between 1-9 (Table 3). Clinical and laboratory characteristics per patient with abnormal flow chamber results are shown in Supplemental table S3. 9/25 (36%) patients with abnormal flow chamber parameters were so-called 'PFA-only' patients, with a prolonged PFA-CT in one or both cartridges. The number of abnormalities in bright field platelet SAC was 10, in DIOC6 Platelet SAC 11, morphology score 5,

contraction score 4, multilayer score 4. On collagen type I, BF platelet SAC was abnormal in 5 patients, $\alpha_{IIb}\beta_3$ was abnormal in 3 patients, PS exposure was abnormal in 5 patients and P-selectin in 8. BF platelet SAC on collagen type III was abnormal in 9 BDUC patients. Comparison of the ISTH-BAT score, vWF activity and antigen levels and PFA-200 results between BDUC patients with and without abnormal flow chamber results showed no statistically significant differences (figure 4).

Table 3: Overview of the abnormalities per parameter in BDUC patients (n = 25).

Patient number (PFA) indicated PFA-only patients. Nr. Number; PFA, platelet function analyzer; BF, bright field; SAC, surface area coverage; col, collagen; $\alpha_{IIb}\beta_3$, integrin activation; PS exp, phosphatidylserine exposure; P-sel, P-selectin.

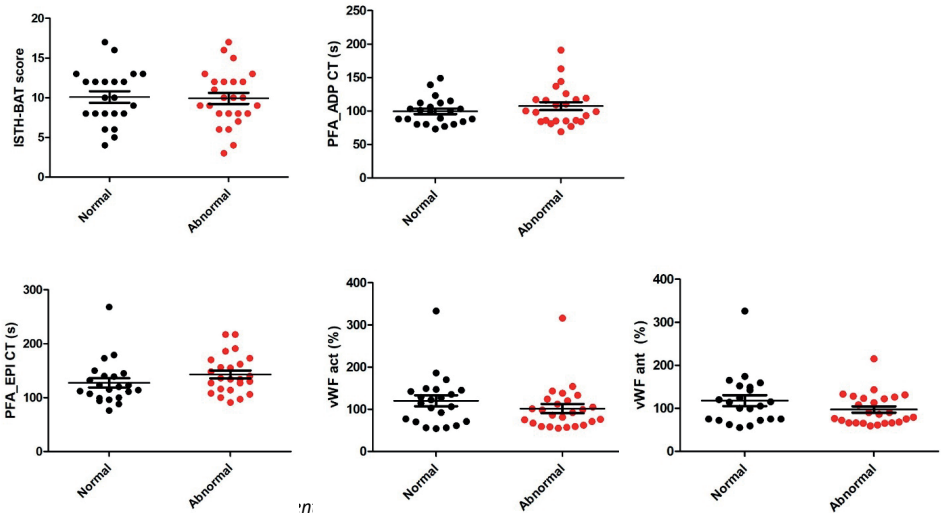

Figure 4: Characteristics in BDUC patients with and without abnormal flow chamber results.

Comparison of ISTH-BAT score, vWF antigen and activity levels and PFA-200 results showed no statistically significant differences between BDUC patients with and without abnormal flow chamber results. The Mann Withney U test was used for comparison of both groups. ISTH-BAT, International society on thrombosis and hemostasis bleeding assessment tool; PFA, platelet function analyzer; EPI, epinephrine; ADP, adenosine diphosphate; VWF act, von Willebrand factor activity; VWF ant, von Willebrand factor antigen.

Correlation analysis and principle component analysis

To explore potential correlations between the microfluidic parameters, clinical characteristics and results from conventional primary hemostasis assays, correlation analysis was performed in the 47 BDUC patients. Results of the correlation coefficients R are visualized as a heatmap with color intensity indicating the strength and direction of correlations (figure 5). A heatmap with the accompanying p-values can be found in supplementary Figure S1. VWF activity and antigen correlated moderately with brightfield

platelet SAC on the collagen III surface (R=0.374, p=0.009; R =0.355, p= 0.013). Moderate correlations were also seen between platelet count and morphology, contraction and multilayer scores (R=0.416, p=0.003; R=0.402, p=0.037; R=0.351, p=0.015).

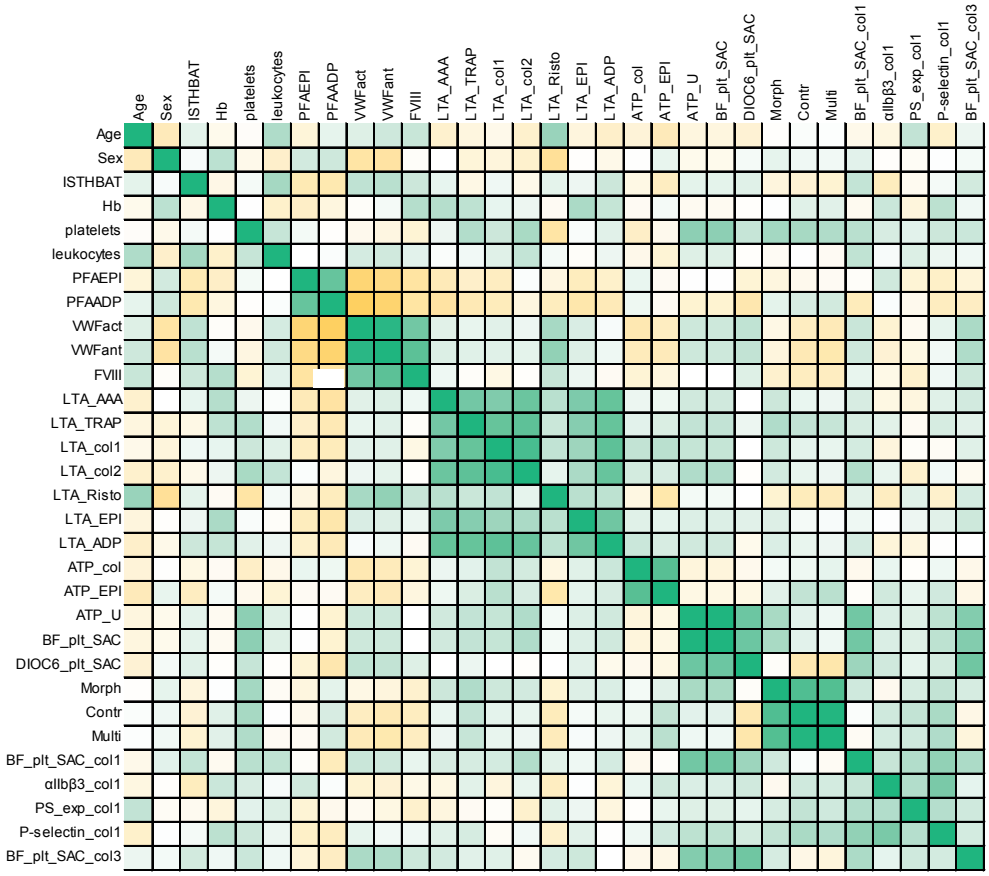

Figure 5: Correlation analysis to investigate associations between microfluidic parameters, clinical characteristics and results from conventional primary hemostasis assays in BDUC patients (n=47).

Heat mapped correlations of microfluidic parameters in 47 BDUC patients. Heat mapped Spearman correlation coefficients R: a darker color indicated a stronger positive (green) or negative (yellow) correlation. Hb, hemoglobin; PFA, platelet function analyzer; EPI, epinephrine; ADP, adenosine diphosphate; VWF act, von Willebrand activity; VWF ant, von Willebrand antigen; Col, collagen; LTA, light transmission aggregometry; AAA, arachidonic acid; TRAP, thrombin receptor activatable peptide aggregometry; risto, ristocetin; ATP, adenosine thriphosphate release; U, thromboxane A2 analogue; BF, brightfield; plt, platelet; SAC, surface area coverage; Morph, morphology score; Contr, contraction score; Multi, multilayer score; $\alpha_{IIb}\beta_3$, fibrinogen binding; PS_exp, PS exposure; Psel, P selectin expression.

DISCUSSION

This study investigated whether a multiparameter microfluidic assay, the Maastricht flow chamber, can identify shear-dependent hemostasis defects in BDUC patients. In more than half of the BDUC patients in this study (53%), one or more abnormalities were observed in the microfluidic parameters on the collagen I and/or collagen III spot, suggestive for a primary hemostasis defect. Fibrin formation on the TF/col I spot showed no significant differences between BDUC patients and healthy volunteers.

Abnormalities in the primary hemostasis have already been extensively studied in BDUC patients using currently available laboratory tests for platelet function and VWF. Per definition, BDUC patients have normal LTA results [5]. The addition of a platelet secretion assay adds another group of platelet function disorder diagnoses, more specifically platelet secretion defects, in a patient population suspected for a MBD [26]. The use of flow cytometry based platelet receptor expression and platelet activation tests also causes a modest increase in diagnostic yield [27]. Normal VWF levels are also required for a BDUC diagnosis [5]. However, VWF levels are known to fluctuate and repeated testing is advised, especially in patients with VWF activity levels <80% [28]. Moreover, in VWD type 1, VWF levels are known to increase with age, possibly leading to a 'false' BDUC diagnosis [29].

Previous studies have investigated thrombus formation under shear conditions in patients with established bleeding disorders, demonstrating that the Maastricht flow chamber assay is capable of detecting known hemostatic abnormalities. One study showed abnormal thrombus formation in patients with platelet function disorders, including Glanzmann's thrombasthenia and grey platelet syndrome, using a flow-based hemostasis assay [10]. Our previous study used the Maastricht flow chamber to evaluate shear-dependent platelet function in patients with an increased bleeding tendency and prolonged PFA closure time as the only aberrant test results. Their results showed lower platelet adhesion and thrombus formation parameters in 14 PFA-only patients, suggesting a shear-dependent platelet function defect [9].

In the current study, 13/47 patients showed abnormalities with respect to platelet surface area coverage (brightfield and/or DIOC6) on the microspot coated with collagen type l, indicative of a potential platelet adhesion defect. In a damaged blood vessel, exposed endothelial collagen initiates the primary hemostasis process and causes platelets to adhere [30]. Low scores could indicate impaired adhesion of platelets to the collagen coated surface. Platelet activation was abnormal in eight patients based on impaired PS-exposure and/or $\alpha_{IIb}\alpha_3$ activation. Abnormalities in parameters $\alpha_{IIb}\alpha_3$ activation, morphology, multilayer and contraction score could be indicative of a problem in platelet aggregation. Lastly, impaired P-selectin expression could indicate a platelet secretion

defect. However, the different hemostatic processes cannot be clearly separated based on the parameters as this is inherent to an integrative assay in which multiple independent processes are measured simultaneously.

Multiple reasons could be the cause for impaired platelet aggregation. It could indicate a defect in platelet activation, reduced granule secretion leading to impaired inter-platelet activation, or abnormalities in plasma proteins, such as fibrinogen or vWF, limiting cross-linking of platelets [18, 19]. In all of these situations, platelets may hypothetically adhere initially but fail to maintain adhesion or contract due to mechanical fragility under flow conditions, explaining why static tests often fail to detect such malfunctions. This highlights the relevance of the Maastricht flow chamber assay as a technique to detect flow related impairments in platelet adhesion and aggregation parameters in BDUC patients.

The abnormalities found in the fluorescent label parameters were measured at a collagen type I surface. Collagen type l is a strong platelet activator primarily engaging the GPVI and integrin $\alpha_2\beta_1$ receptors, leading to adhesion and activation. When looking at the fluorescent label measurements, abnormalities were found for PS exposure on the collagen type l spot in five BDUC patients. Impaired PS exposure could potentially result from defective GPVI signaling, which plays a key role in platelet activation and granule secretion [19]. P-selectin expression was also impaired on the collagen type l spot in nine BDUC patients. According to one study, a high shear-stress environment is necessary for vWF-induced P-selectin translocation through vWF-GPIbα signalling [31]. The impaired P-selectin expression found in the BDUC patients could possibly indicate a defect in the VWF-GPIbα signalling pathway. Furthermore, the platelet surface area coverage measured on the collagen type lll spot was found to be reduced in nine BDUC patients. As collagen type lll has a higher affinity for VWF, this reduction could also possibly indicate any impairment in the vWF–GPIb-mediated initial platelet adhesion pathway [32], which cannot be detected with the static VWF antigen and activity tests. When VWF is not working properly, it could lead to impaired platelet adhesion and therefore reduced platelet deposition and thrombus formation [33]. One study screened for von Willebrand factor deficiencies with a custom microfluidic flow assay and found abnormalities in patients with low VWF and type 1 VWD, confirming the ability of this model to identify VWF related abnormalities [34].Taken together, these results possibly mean that a specific receptor pathway (GPVI or $\alpha_2\beta_1$), the VWF-A1 interaction, or a regulatory inhibitory mechanism is partially impaired in (part of) BDUC patients.

The additional level of detail provided by the Maastricht flow chamber allows the identification of clinically meaningful abnormalities that can support a diagnosis of BDUC with indications of a flow-related platelet defect, unlike the PFA-200 which by itself never

leads to a definitive platelet function disorder diagnosis. While treatment strategies are not altered based on these findings, providing patients with a more defined explanation for their bleeding symptoms can be of considerable value. A diagnosis that offers direction and acknowledgement can help patients better understand their condition and may reduce uncertainty and frustration associated with an otherwise unexplained bleeding tendency.

There are several limitations to this study. First, patients were selected from the ProBe-AHP study based on an elevated ISTH-BAT score and normal standard laboratory hemostasis tests, which could have introduced selection bias by favoring patients with more severe bleeding phenotypes. Another limitation is the lack of a direct comparison to validated or alternative platelet function assays, which makes correlations to clinical outcomes less robust. Repeated measurements in larger cohorts may help address this limitation if similar results are consistently observed.

Future research should address the practical implementation of the Maastricht flow chamber, as it currently requires substantial time and highly specialized personnel. While this does not limit the validity of the present study, it represents a potential hurdle for routine diagnostic use. In this context, the use of commercially available microfluidic platforms, such as the total-thrombus analysis system (T-TAS) or Anysis, could be explored, although this may come at the expense of the detailed resolution provided by the Maastricht flow chamber [35, 36].

Further investigation of the observed abnormalities is warranted to elucidate the underlying mechanisms of bleeding tendency. This could include electron microscopy, targeted genetic analyses, proteomics, and additional VWF studies. Another avenue for future research is the integration of endothelial cells into the Maastricht flow chamber model to better mimic in vivo conditions, as endothelial dysfunction may contribute to bleeding in a subset of BDUC patients [37-39]. Finally, the diagnostic value of other, less labor-intensive microfluidic models in BDUC patients could also be explored.

In conclusion, this study showed that the Maastricht Flow chamber assay can identify 53% of BDUC patients with flow-dependent abnormalities in the primary hemostasis. The Maastricht flow chamber provides more detailed insights into flow-dependent platelet abnormalities and can support a flow-related platelet defect diagnosis. To really understand the underlying platelet function defect, extensive research is still required. Although the diagnosis may not change the treatment regimen, it could offer meaningful clinical direction and recognition for the patient.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1: Overview of hemostasis tests performed in patients from the ProBe-AHP study.

Screening hemostasis tests	Complete blood count, PT, aPTT, fibrinogen
Von Willebrand tests	vWF activity and vWF antigen
Coagulation factors	FII, FV, FVII, FVIII, FIX, FX, FXI, FXIII
Platelet function tests	PFA-200, LTA, ATP-release

PT, prothrombin time; aPTT, activated partial thromboplastin time; vWF, von Willebrand Factor; F, factor; PFA, platelet function analyzer ; LTA, light transmission aggregometry; ATP, adenosin triphosphate.

Supplementary Table S2: Reference values for Maastricht flow chamber parameters based on the 2.5th - 97.5th percentiles of measurements in 49 healthy volunteers.

Maastricht Flow chamber parameter	**Reference interval (2,5th – 97,5th percentiles) [min – max range]**
%SAC brightfield (7 min.)	49-78 [46-81]
%SAC DIOC6 (7 minutes)	35-56 [30-57]
Morphology score (7 minutes)	4.5-6 [4-6]
Contraction score (7 minutes)	2.5-4 [2-4]
Multilayer score (7 minutes)	2.5-4 [2-4]
%SAC brightfield col I (10 minutes)	44-78 [33-82]
%SAC $\alpha_{IIb}\beta_3$ activation col I (10 minutes)	7-34 [1-38]
%SAC PS-exposure col I (10 minutes)	5-24 [5-25]
%SAC P-selectin expression col I (10 min.)	27-53 [21-57]
%SAC brightfield col III (10 min.)	64-92 [50-92]

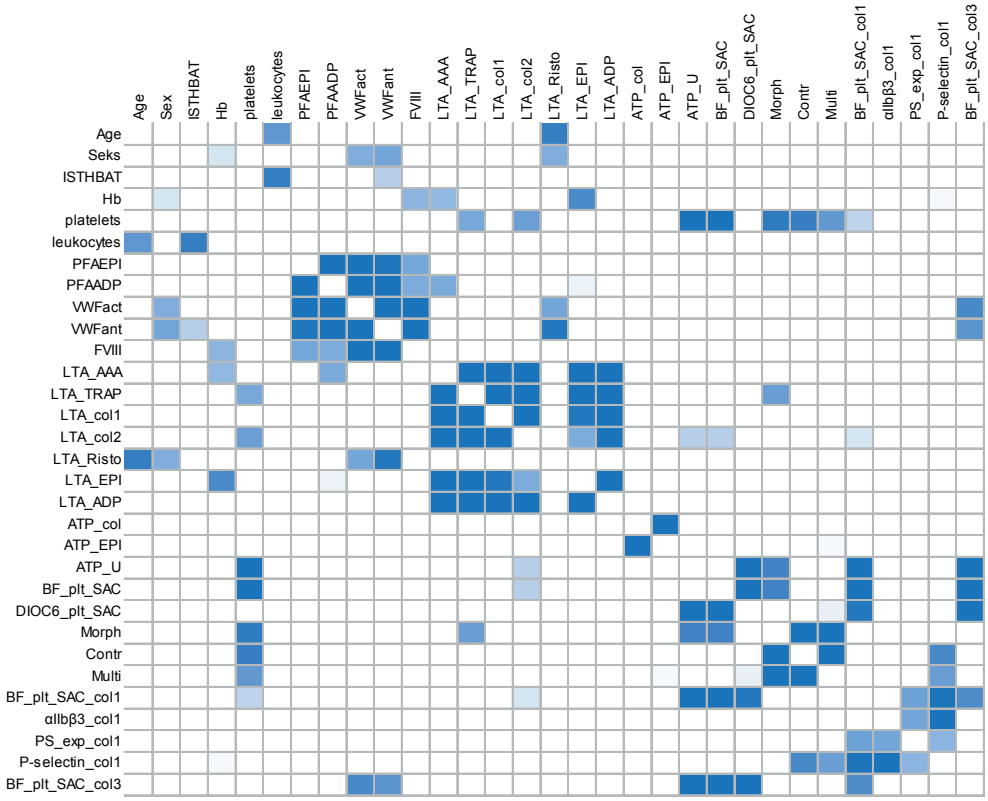
%SAC, percentage of surface area coverage; col I, collagen type 1; col III, collagen type 3; PS, phosphatidylserine.

Supplementary Table S3: Clinical and laboratory parameters per BDUC patient with abnormal flow chamber results (n=25).

	Age	ISTH-BAT	VWF-act	VWF-ant	VWF-ratio	PFA_EPI	PFA_ADP
Reference ranges		**<6 (women)** **<4 (men)**	**50-150 %**	**50-150%**	**>0.7**	**84-160 s**	**71-118 s**
1	70	8	92	113	0.8	**173**	**163**
2	44	10	**58**	66	0.9	**162**	**144**
3	59	9	112	131	0.9	134	100
4	37	17	154	133	1.1	100	93
5	54	3	**59**	65	0.9	**217**	**191**
6	54	12	**57**	**59**	1.0	-	-
7	29	13	86	75	1.1	116	84
8	50	4	76	86	0.9	**186**	117
9	22	7	62	66	0.9	155	**137**
10	25	13	138	143	1.0	**217**	69
11	36	12	143	122	1.2	97	85
12	19	9	99	76	1.3	156	110
13	20	6	98	90	1.0	127	86
14	66	15	316	215	1.4	106	81
15	35	6	67	61	1.1	114	84
16	48	8	101	90	1.1	140	109
17	34	16	**55**	68	0.8	108	86
18	52	12	**59**	65	0.9	**170**	119
19	33	8	75	66	1.1	130	98
20	37	8	124	123	1.0	91	77
21	29	10	105	108	1.0	134	117
22	52	12	**59**	65	0.9	**170**	**119**
23	56	11	81	79	1.0	127	99
24	31	9	71	72	1.0	**191**	**126**
25	26	10	120	128	0.9	136	85

Prolonged PFA-CT's are indicated in bold (black). Von Willebrand factor levels between 50-60% are indicated in bold (dark blue).

ISTH-BAT, International society of thrombosis and hemostasis bleeding assessment tool; vWF act, von Willebrand factor activity; vWF ant, von Willebrand factor antigen; PFA_EPI, platelet function analyzer epinephrine; PFA_ADP, platelet.



Supplementary Figure S1: p-values accompanying correlation coefficients.

Heat mapped p-values in which a darker color blue indicates a more significant correlation. Hb, hemoglobin; PFA, platelet function analyzer; EPI, epinephrine; ADP, adenosine diphosphate; VWF act, von Willebrand activity; VWF ant, von Willebrand antigen; Col, collagen; LTA, light transmission aggregometry; AAA, arachidonic acid; TRAP, thrombin receptor activatable peptide aggregometry; risto, ristocetin; ATP, adenosine thriphosphate release; U, thromboxane A2 analogue; BF, brightfield; plt, platelet; SAC, surface area coverage; Morph, morphology score; Contr, contraction score; Multi, multilayer score; $\alpha_{IIb}\beta_3$, fibrinogen binding; PS_exp, PS exposure; Psel, P selectin expression.



CHAPTER 9

CASE REPORT

a family with hypodysfibrinogenemia and a novel FGG mutation

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ABSTRACT

Background: Hypodysfibrinogenemia is a rare congenital fibrinogen disorder (CFD) which may induce thrombotic and bleeding events. Therefore, patient management needs careful evaluation. Routine coagulation tests are inadequate to predict the clinical phenotype.

Clinical findings: A 60-year-old woman with both bleeding and thrombotic complications and her two daughters were referred to our center for genotypic and phenotypic analysis of a CFD.

Diagnosis: Conventional laboratory results led to the diagnosis of hypodysfibrinogenemia in all three subjects. They all carried the same heterozygous c.1124A>G mutation in *FGG* resulting in p.Tyr375Cys amino acid substitution, which was confirmed by protein variant analysis from plasma. *In silico* structure analysis predicted possible conformational and functional changes of the fibrinogen molecule. Thrombin generation indicated a hypercoagulable state confirmed by microfluidics that showed enhanced fibrin formation in both daughters, regardless of the coagulation trigger.

Conclusion: We report on a family with hypodysfibrinogenemia and a novel *FGG* heterozygous missense mutation, possibly leading to conformational changes or covalent dimerization. Thrombin generation and particularly microfluidic measurements disclosed a hypercoagulable state, which was not detected with routine coagulation tests, justifying a different patient management.

INTRODUCTION

Congenital fibrinogen disorders (CFDs) are rare and clinically heterogeneous haemostatic disorders that can be classified as quantitative (type I) or qualitative (type II) (1, 2). The associated clinical phenotype ranges from bleeding to thrombosis and cannot be easily predicted from routine laboratory test results, especially for qualitative defects (1-5). This makes the management of these patients complex. Follow-up is recommended even in asymptomatic patients, as they are prone to developing both thrombotic and bleeding events (3).

In the last years, global haemostatic assays such as the viscoelastic ROTEM test, thrombin generation and thrombus formation in the Maastricht flow chamber, have been developed to assess the overall hemostatic balance. These assays could provide information on the bleeding or thrombotic tendency in patients with CFDs (6-8). Here we present a family with congenital hypodysfibrinogenemia associated with a novel *FGG* gene mutation, and we report on the performance of global hemostasis assays in elucidating the clinical phenotype.

CASE REPORT

A 60-year-old woman, who was diagnosed with congenital hypofibrinogenemia in another centre at the age of 26 years, was referred to our hemophilia treatment center (HTC) for a second opinion. Her medical history was remarkable for three spontaneous miscarriages. The patient underwent the first spontaneous abortion at 22 weeks gestation when she was 26 years old. This miscarriage was caused by a placental infarction, followed by a major fluxus during partus, for which a blood transfusion was required. Subsequent analysis for bleeding tendency uncovered low fibrinogen levels, but no other abnormalities predisposing to bleeding. A diagnosis of congenital hypofibrinogenemia was made at that time. Shortly after the first miscarriage, the patient presented with pulmonary embolism (PE).

Two additional spontaneous abortions occurred at 13 weeks gestation at the age of 27 years and 28 years, respectively. After these three consecutive miscarriages, during the next pregnancies she received prophylactic treatment with cryoprecipitate two times a week and therapeutic low-molecular weight heparin (LMWH). This resulted in two successful pregnancies, out of which two daughters were born. Both daughters were tested immediately after birth and diagnosed with hypofibrinogenemia. No further bleeding or thrombotic events occurred in the proband until the age of 59 years, when she presented with a second episode of spontaneous PE. Anticoagulant therapy with dabigatran was

started. After this second thrombotic event, the patient and her two daughters were referred to our HTC for genotypic and phenotypic evaluation, therapeutic advice in case of bleeding or surgical or dental interventions, and guidance for future pregnancy and delivery (daughters).

The daughters of the proband were 31 (daughter 1) and 23 (daughter 2) years old upon referral. Daughter 1 suffered from menorrhagia, which normalized after initiation of an oral contraceptive. She had not been challenged by surgical interventions, dental extractions, pregnancies, or deliveries yet. The International Society on Thrombosis and Haemostasis bleeding assessment tool (ISTH-BAT) score at the time of referral was 2. There was no history of thrombotic events. Daughter 2 had no history of bleeding tendency or thrombotic events and her ISTH-BAT score was 0. She also used an oral contraceptive at the time of referral. The ISTH-BAT score of the proband was 3.

Approximately one year after the proband was referred to our HTC, she presented with spontaneous PE (third episode) whilst being treated with dabigatran. About six months earlier, the level of dabigatran was 58 ng/mL, measured two hours after dabigatran intake. Taking into consideration the dosage and the time of administration, this level was just below the expected range of 60-450 ng/mL (9) which might have played a role in the PE recurrence. Anticoagulant treatment was switched to fenprocoumon combined with therapeutic LMWH as bridging therapy until the INR was adequate. The timeline of the proband's medical history is schematically presented in Figure 1.

DIAGNOSTIC ASSESSMENT

Diagnostic testing

Conventional laboratory testing

Conventional laboratory test results are shown in Table 1. The prothrombin time (PT) was prolonged and reptilase time was outside the reference range in all three subjects. In the proband, fibrinogen activity was decreased whilst fibrinogen antigen levels were within reference ranges. Differently, both fibrinogen antigen and activity levels were decreased in the daughters. Fibrinogen test results in all three subjects point in the direction of hypodysfibrinogenemia (fibrinogen activity/antigen ratio <0.7). They classify as mild hypodysfibrinogenemia (type 4C) as their fibrinogen antigen levels are between 1 g/L and the lower limit of the normal range (1). The higher fibrinogen antigen levels in the proband compared to her daughters might be explained by the age-dependent increase of fibrinogen antigen levels (19).

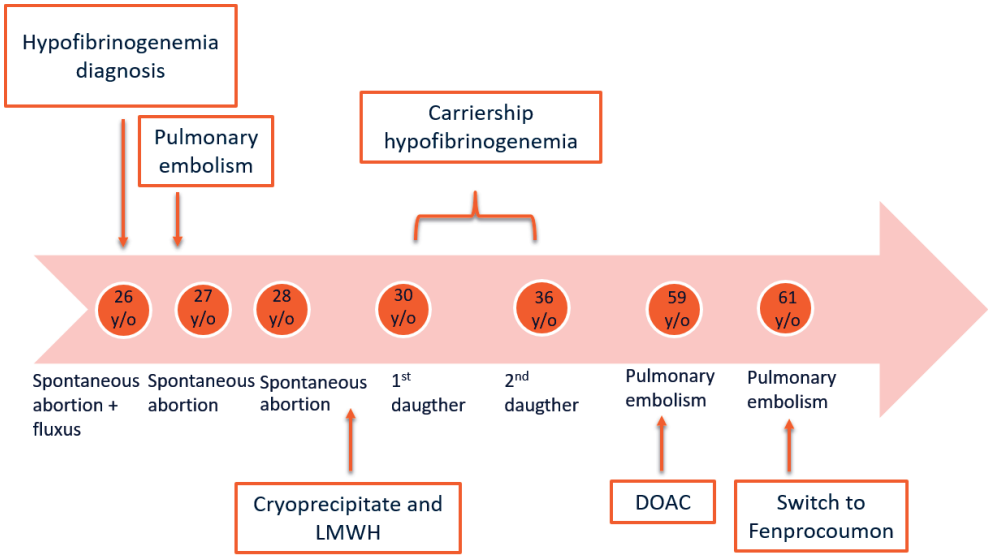

Figure 1: Timeline of the episodes of care of the proband.

Plasminogen levels were slightly elevated in both daughters, whereas factor XIII (FXIII) activity levels were mildly decreased. This is probably a test artefact due to low fibrinogen levels, as described in the Siemens Healthineers Berichrom FXIII manual (10).

Factor V Leiden and Prothrombin G20210A were absent. Activated protein C (APC) resistance, protein C activity, total and free protein S antigen, and lupus anticoagulant were all within the normal range (data not shown). In the proband, thrombophilia workup was done under dabigatran as this treatment could not be discontinued. For these assays, DOAC-remove was used before carrying out the measurements.

DNA sequence analysis

DNA sequence analysis of the *FGA* and *FGB* genes showed no abnormalities. Analysis of the *FGG* gene showed the presence of a novel heterozygous variant (c.1124A>G) predicting the p.Tyr375Cys amino acid substitution in all three subjects. This variant is located in a functional domain of the *FGG* gene in which multiple pathogenic missense variants have been reported (11). This variant was classified as likely pathogenic (class 4), based on the American College of Medical Genetics and Genomics (ACMG) variant classification criteria (12).

Fibrinogen protein analysis

The presence of this missense mutation at the protein level was confirmed by mass spectrometry (LC-MS/MS) analysis of plasma fibrinogen (Figure 2). Both fibrinogen ɣ

Table 1: Demographic and clinical characteristics, and results of clinical laboratory measurements in the proband and daughters.

Laboratory Measurements	Normal range	Proband	Daughter 1	Daughter 2
Age (years)		60	31	23
Gender		Female	Female	Female
Medication		Dabigatran	Oral contraceptive	Oral contraceptive
Clinical features		Haemorrhagic and thrombotic events	Menorrhagia	Asymptomatic
Phenotype		Hypodysfibrinogenemia	Hypodysfibrinogenemia	Hypodysfibrinogenemia
ISTH-BAT score	0-6	3	2	0
Thrombin time (s)	15-22	*	18.9	**22.2**
Prothrombin time (s)	9.9-12.4	**13.4**	**14.6**	**
Reptilase time (s)	17-25.4	**26.9**	**27.9**	**28.6**
Fibrinogen Antigen (g/L)	1.8-3.0	1.87	**1.25**	**1.04**
Fibrinogen Activity (g/L)	1.7-4.0	**0.8**	0.6	0.5
Fibrinogen activity to antigen ratio		0.4	0.5	0.5
Factor XIII activity (%)	70-140	76	**55**	**51**
Plasminogen activity (%)	80-120	***	**137**	**131**
Genotype		Heterozygous	Heterozygous	Heterozygous
Gene		*FGG*	*FGG*	*FGG*
Nucleotide		c.1124A>G	c.1124A>G	c.1124A>G
Amino acid		p.Tyr375Cys	p.Tyr375Cys	p.Tyr375Cys

Results that are outside the reference ranges are marked in bold.
* Not reliable due to dabigatran treatment
**Prothrombin time could not be measured due to very low levels of fibrinogen.
***Not measured

chain-derived peptide sequences, comprising a tyrosine (Tyr) or cysteine (Cys) at position 375, were identified in plasma from the proband and her daughters, whereas only the wild-type p.Tyr375 sequence was detected in control plasma.

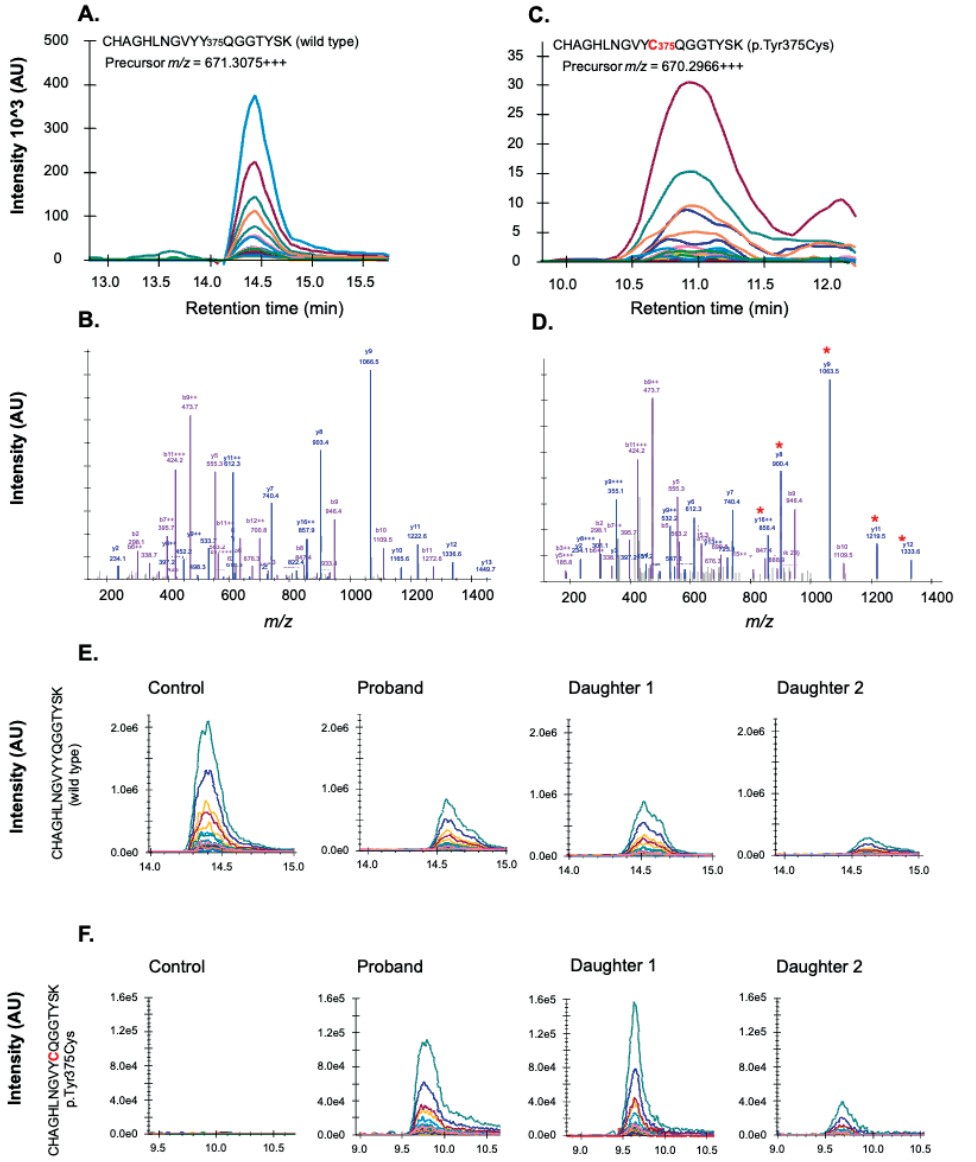

Figure 2: Confirmation of p.Tyr375Cys amino acid substitution by mass spectrometry analysis of fibrinogen ɣ chain from plasma.

Peptides unique to wild-type (A&B) or variant (C&D) fibrinogen ɣ chain were quantified in plasma. LC chromatogram and mass spectra shown with product fragments unique to the variant sequence indicated by asterisk. (E) Tryptic peptide representing wild-type fibrinogen ɣ chain in pooled normal plasma (control) and plasma of studied family members. (F) Tryptic peptide representing variant p.Tyr375Cys measured in pooled normal plasma (control) and plasma of studied family members.

The newly introduced cysteine residue is located near the A-hole, a functional site involved in fibrin polymerisation (13). As indicated by *in silico* analysis of the crystal structure of fibrinogen (PDB ID 3GHG) and molecular dynamics (MD) simulations, this free cysteine (p.Tyr375Cys corresponding to Tyr349 residue in the PDB structure) could form spurious disulfide bonds with cysteines 339 and 326 of the same ɣ chain, causing a significant conformational change of the A-hole that could affect fibrin polymerisation (supplementary Figure S1). Alternatively, Cys375 could promote disulfide bonding between mutant ɣ chains, resulting in grossly abnormal fibrinogen molecules that are unlikely to be secreted. Accordingly, the 375Cys ɣ chain variant was strongly under-represented in plasma of the three mutation carriers (as assessed by mass spectrometry) and no structural abnormality could be detected by Western blot analysis of their plasma fibrinogen (data not shown). Taken together, these observations could account for the hypodysfibrinogenemia observed in carriers of the *FGG* p.Tyr375Cys mutation.

Approximately 10% of all fibrinogen molecules contain a splicing isoform of the ɣ chain, known as ɣ' chain, that mediates high-affinity binding of fibrinogen to thrombin and inhibits thrombin-catalyzed activation of platelets, FV and FVIII (14-16). Since the fibrinogen ɣ' fraction has been inversely associated with the risk of venous thrombosis (17), we also measured fibrinogen ɣ' levels. ELISA assays showed that fibrinogen ɣ' levels in the mother (63.5% of pooled normal plasma), in daughter 1 (44.0%) and in daughter 2 (32.8%, measured after discontinuation of the pill) were decreased in proportion to their total fibrinogen levels, indicating that the identified *FGG* variant does not alter the ratio between the two fibrinogen ɣ chain isoforms.

Thrombin generation

Thrombin generation (Calibrated Automated Thrombography (18) in platelet-poor plasma) could only be measured in the daughters, because the proband was on anticoagulant treatment. Both daughters were using oral contraceptives at the time of testing, but daughter 2 was also re-assessed after discontinuation of this medication. At low tissue factor (TF), both daughters showed slightly shorter lag times and higher peaks of thrombin generation than pooled normal plasma, indicating a mild hypercoagulable state. However, this disappeared when daughter 2 was re-tested off the pill. When thrombin generation was measured at high (10 pM) TF and soluble TM was added to probe the protein C pathway, both daughters showed mild APC resistance with residual endogenous thrombin potential (ETP) of 72% and 60% respectively, vs. 42% in pooled normal plasma. Interestingly, although the residual ETP in daughter 2 decreased to 51% after discontinuation of oral contraceptive use, it did not normalize completely (supplementary Figure S2).

Microfluidic assay

Both daughters were also tested in a microfluidic assay of thrombus formation under flow using the Maastricht Flow Chamber (Figure 3). In this assay, whole blood was perfused over surfaces coated with collagen type III or collagen type I with or without TF, and platelet deposition and fibrin formation were monitored using fluorescent probes (19). Platelet deposition on the collagen surfaces was similar between both daughters and controls, whereas the aggregate coverage on the relatively weak agonist collagen type III was remarkably higher in the daughters (supplementary Figure S3). In control blood, fibrin was solely formed on the surface where collagen type I was co-coated with TF. In contrast, in the daughters' blood, fibrin formation was detected on all surfaces, regardless of TF triggering of coagulation (Figure 3). Additional data on surface area coverage (%SAC) over time can be found in supplementary Figure S3. Although both daughters were using oral contraceptives at the time of testing, re-assessment of daughter 2 when she was no longer using oral contraceptives (after three months) yielded similar results to the first measurement, suggesting a minor contribution of oral contraceptive use to the procoagulant effects observed in this assay.

THERAPEUTIC INTERVENTION AND FOLLOW-UP

Based on the extensive geno- and phenotyping, a personal treatment plan was set-up for all three subjects. For the proband, the treatment plan included advice concerning prophylaxis and treatment for both thrombotic and hemorrhagic risk situations. In case of bleeding or a surgical intervention, administration of fibrinogen concentrate with a target value of fibrinogen activity level of >1.5 g/L is recommended. In case of life-threatening hemorrhage, a target value of fibrinogen activity of >2 g/L should be used. Concerning antithrombotic therapy, the advice was to bridge fenprocoumon with LMWH in case of a surgical intervention. Anticoagulant treatment in patients with CFDs is in concordance with the recommendations for the general population (4, 20). Direct anticoagulants are the treatment of first choice, LMWH is the treatment of second choice. Vitamin K antagonists can be used as well, but can be difficult to monitor as a pre-existing PT prolongation can influence the INR, complicating the monitoring of the target INR value (4, 20). Our proband had a normal pre-existing INR and treatment with fenprocoumon was chosen because of recurrent PE under dabigatran treatment.

For both daughters, a similar treatment plan was set up concerning management in case of bleeding and dental and/or surgical interventions. Guiding advice during pregnancy and delivery was added. In case of pregnancy, it was recommended to start with a prophylactic dose of LMWH. During delivery, a target value of fibrinogen activity of >1.5 g/L is recommended. In case of post-partum hemorrhage, a target value of fibrinogen

activity of >2.0 g/L is recommended, including treatment with fibrinogen concentrate. Taking into consideration the prothrombotic risk of oral contraceptives, the proband's daughters were advised to switch to another form of contraception, such as the use of an intrauterine device (IUD) (21, 22).

DISCUSSION

The diagnostic workup of CFDs is relatively straightforward, but managing these patients is challenging as they can present with both bleeding and thrombotic manifestations. While a prolonged PT and low fibrinogen activity might indicate a bleeding tendency, conventional laboratory tests do not provide any indications for a thrombotic diathesis. This case report illustrates the utility of specialized research hemostasis tests, such as thrombin generation and thrombus formation under flow, in elucidating the clinical phenotype in three members of a hypodysfibrinogenemic family with a novel *FGG* mutation.

Thrombin generation at low TF showed slightly shorter lag times and higher peak heights in both daughters compared to normal pooled plasma, which was largely due to oral contraceptive use at the time of testing. Remarkably, however, the thrombin peak measured in the plasma of daughter 2 after discontinuation of the pill was still similar to that of normal plasma, whereas her fibrinogen antigen level (~1 mg/mL) would predict a lower peak height (8, 23, 24). Whether this discrepancy is attributable to qualitative properties of her mutant fibrinogen or to other determinants of thrombin generation in her plasma remains to be elucidated. Based on thrombin generation at high TF in the absence and presence of TM, both daughters also had APC-resistance, which persisted in daughter 2 after discontinuation of oral contraceptive use. This may be explained, at least in part, by her low fibrinogen level, as fibrinogen (and particularly fibrinogen γ') have been shown to counteract APC resistance (25). Overall, our findings seem in line with a previous study of thrombin generation performed with ST Genesia® in 22 patients with CFDs, where peak height and ETP were found to be inversely correlated with fibrinogen activity in both the STG-BLS (low TF) and STG-TS-TM (higher TF with TM) variants of the assay (6).

Microfluidic testing was performed to further investigate the overall hemostatic profile under shear. Two remarkable findings of these experiments were (i) a higher aggregate coverage on the collagen III spot in both daughters and (ii) fibrin formation on all three spots in both daughters, even in the absence of extrinsic triggering of coagulation. This is noteworthy, because in healthy controls, fibrin formation is only observed on the collagen I spot co-coated with TF. These results indicate a hypercoagulable rather than a hypocoagulable state in both daughters (without clinical manifestations to date). Fibrin formation on the collagen I spot and the collagen III spot is not expected in our flow model,

since extrinsic activation with TF is needed to induce the coagulation cascade. The findings in both daughters are unlikely to be artefacts, as they were not observed in the controls, which were measured under the same circumstances. Moreover, repeated microfluidic testing in daughter 2, three months after stopping the use of oral contraceptives, showed similar results, indicating reproducibility over time despite discontinuation of oral contraceptives.

Unfortunately, thrombin generation and microfluidic assays are rarely available in clinical settings and their poor automatization and standardization still limits their clinical applicability. Another major drawback is that they do not probe fibrinolysis, abnormalities of which may contribute to both bleeding and thrombotic tendencies.

Besides its procoagulant function as a precursor of fibrin, fibrinogen has been reported to exert an anticoagulant activity known as "antithrombin I" (26). The main effector of this activity is the fibrinogen γ' fraction, which has been shown to bind thrombin with high affinity and to inhibit its ability to activate platelets, FV and FVIII (14-16). The physiological relevance of these effects is supported by the association of the common *FGG* H2 haplotype and other genetic variants predicting relatively lower fibrinogen γ' levels with an increased risk of venous thrombosis (17, 24). Therefore, it is possible that the lower levels of fibrinogen (γ') in the family under study promotes a hypercoagulable state (26, 27). However, since not all hypo(dys)fibrinogenemias are associated with venous thrombosis, as was also observed in a recently published study on CFD's (28), it is likely that other qualitative properties of fibrinogen conferred by the *FGG* p.Tyr375Cys mutation also contribute to a prothrombotic state, possibly by altering cloth strength, structure or stability (27). Additional studies specifically focusing on clot formation, structure and degradation would be required to address this issue and to shed more light on the prothrombotic mechanisms of this particular fibrinogen variant.

CONCLUSION

In summary, this case report describes a family with three cases of hypodysfibrinogenemia, caused by a p.Tyr375Cys substitution in the fibrinogen gamma chain and characterized by a prothrombotic tendency. In vitro, a hypercoagulable state was exhibited. Hypodysfibrinogemia can induce both thrombotic and bleeding phenotypes, which emphasizes the importance of understanding the functional defects on the molecular level. In this case, the thrombin generation assay and particularly thrombus formation measurements using the Maastricht Flow Chamber detected a hypercoagulable state in both daughters and changed their treatment plan. The combination of quantitative and functional laboratory tests may have the potential to bridge the gap between the genotype and clinical phenotype in future.

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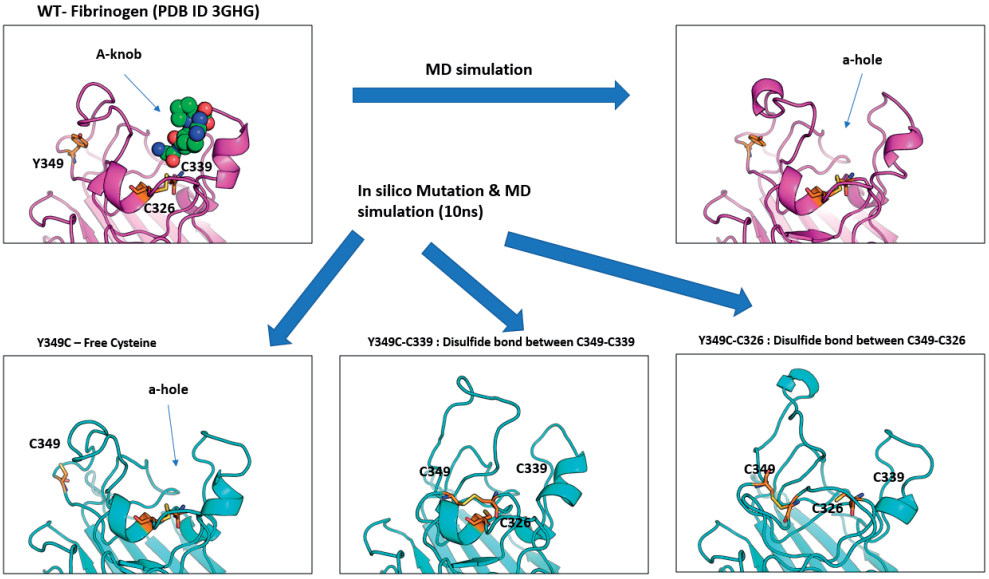
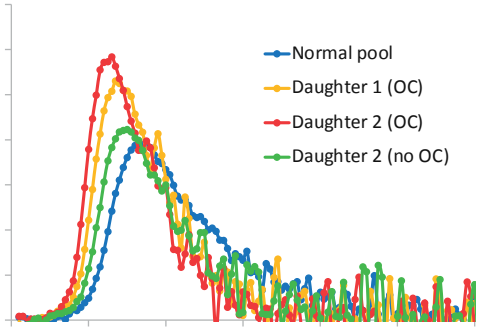
SUPPLEMENTARY MATERIALS



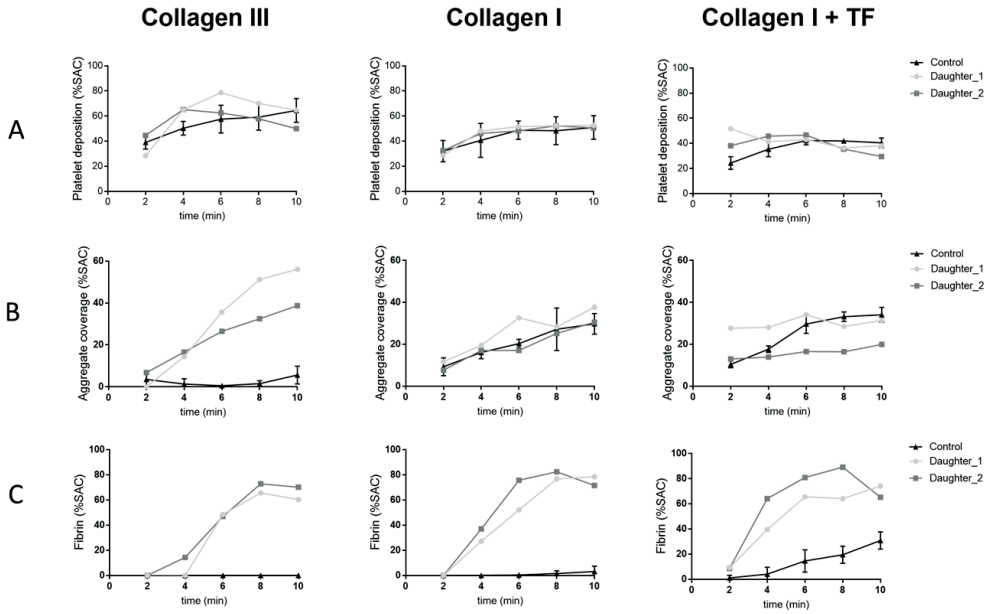
Supplementary Figure S1: In silico analysis of the FGG p.Tyr375Cys mutation.

A) Crystal structure of human fibrinogen (PDB 3GHG). The fibrinogen α-chain, β-chain, and γ-chain are shown in green, cyan and magenta, respectively. The residue affected by the mutation (FGG p.Tyr375Cys), which corresponds to Tyr(Y)349 in the PDB structure, is displayed in orange stick. The residue IDs are labelled according to the PDB structure (3GHG).
B) In silico mutation and molecular dynamics (MD) simulation of the wild-type fibrinogen (PDB ID 3GHG) (top) and the p.(Tyr375Cys) amino acid substitution (bottom). The p.(Tyr375Cys) mutation can lead to three possible scenarios: free cysteine at position C349 (bottom left), a disulfide bond between C349 and C339 (bottom middle), and a disulfide bond between C349 and C326 (bottom right).



Supplementary Figure S2. Left: Thrombin generation was measued in PPP using the CAT-method after initiation of coagulation with 2 pM tissue factor (TF). **Right:** Thrombin generation was measured at 10 pM TF in the presence of increasing concentrations (0-20 nM) of soluble thrombomodulin (sTM). The ratio between the endogenous thrombin potentials (ETP) determined in the presence and absence of TM (expressed in %) was taken as a measure of activated protein C (APC) resistance.

TF: tissue factor; APC: activated protein C; OC: oral contraceptives.



Supplementary Figure S3: Platelet deposition, aggregate coverage and fibrin formation are quantified as percentage of surface area coverage (%SAC) and obtained from images taken at t=2, 4, 6, 8 and 10 minutes. Data obtained from daughters 1 and 2 were compared to 6 healthy donors. Control data are mean ± SD. Panel A: Platelet deposition in daughter 1 and 2 on oral contraceptive. Panel B: aggregate coverage in daughter 1 and 2 on oral contraceptive. Panel C: fibrin formation in daughter 1 and 2 on oral contraceptive.



CHAPTER 10

GENERAL DISCUSSION

GENERAL DISCUSSION OF MAIN FINDINGS AND FUTURE PERSPECTIVES

Sketching the puzzle and piecing it together!

INTRODUCTION

A large variety of hemostasis laboratory assays is available to test for (mild) bleeding disorders [1-3]. However, in over half of the patients suspected for a (mild) bleeding disorder, these assays fail to explain their bleeding tendency [4-6]. Patients that remain undiagnosed after extensive laboratory testing are classified as having a bleeding disorder of unknown cause (BDUC). It is generally assumed that BDUC consists of different subpopulations of patients with fibrinolytic disorders, vessel wall abnormalities and shear-dependent platelet defects amongst others [7-9]. Because of the lack of knowledge on the underlying pathophysiological bleeding mechanisms, there is also a lack of clear guidelines on treatment and management of BDUC patients, which at its turn leads to insecurity and impaired quality of life [10-12]. The absence of clear guidelines also contributes to considerable heterogeneity in the clinical management of BDUC patients across treatment centers. Therefore, this thesis aimed to characterize the BDUC population and to uncover the pathophysiological bleeding mechanisms using advanced hemostasis assays.

In recent years, there has been a growing interest in BDUC, whereas previously it received little attention. In clinical research the characterization of BDUC by establishing a consensus definition remained one of the main goals. Also, the diagnostic work-up and the underlying pathophysiological bleeding mechanisms was identified as other important knowledge gaps (**chapter 2**).
In this thesis all these aspects of BDUC research were addressed. A BDUC definition was formulated based on results of a national online survey that was conducted amongst hemostasis experts from different hemophilia treatment centers, and in concordance with the internationally published BDUC definition (**chapter 3**). To investigate the underlying bleeding mechanisms, different global and innovative hemostasis assays, the tPA-ROTEM (**chapter 5**), thrombin generation assay (TGA) (**chapter 6**), specialized platelet function assays (**chapter 7**) and the Maastricht flow chamber (**chapter 8**), were applied in BDUC patients. Unlike the standard hemostasis assays, which are static and test for a specific part of the hemostatic process, more global hemostasis assays were investigated, integrating different aspects of hemostasis in one test and incorporating physiological elements such as shear rates and body temperature. With the use of these global hemostasis assays, abnormalities were found in a part of the BDUC population in whom

all standard hemostasis assays had failed to show deviant results. Our findings suggest that some global hemostasis assays can be helpful in finding a cause for the bleeding in BDUC patients, or at least point us in a direction to further focus on.

Combined, the chapters of this thesis showed that in a heterogeneous group of BDUC patients, different advanced hemostasis assays can identify defects in some BDUC patients, emphasizing the need for an individualized approach in this patient population. This final chapter summarizes the main findings and elaborates on future perspectives.

PART I: SKETCHING THE PUZZLE: CLINICAL AND METHODOLOGICAL PERSPECTIVES ON BDUC

An illustrative case

A 30-year-old woman was referred by her general practitioner to a hematologist for evaluation of recurrent bleeding symptoms and was subsequently discussed in a multidisciplinary team meeting attended by hematologists, nurse practitioners, and clinical chemists. Her ISTH-BAT score was 13, based on spontaneous hematomas, epistaxis, gingival bleeding, heavy menstrual bleeding, postpartum hemorrhage, and excessive bleeding after dental extractions. She reported that her two-year-old son also frequently developed spontaneous hematomas, and her grandmother had died from surgical bleeding. No formal bleeding disorder diagnosis was known within the family. Standard laboratory hemostasis assays yielded normal results, leading to a diagnosis of BDUC. An empirical treatment plan was provided, including tranexamic acid, desmopressin, and platelet transfusion in case of severe bleeding. Despite the comprehensive evaluation and proposed management plan, the patient remained deeply concerned. She felt uncertain because her physician could not provide a clear explanation for the cause of her bleeding or a definitive treatment strategy. Her worries extended to her young son, who was already showing bleeding symptoms at an early age, and she expressed particular anxiety about the potential risks of a future pregnancy and whether another child might also be affected.

This case represents a typical presentation of a BDUC patient frequently discussed in our multidisciplinary meetings and underscores the many uncertainties which continue to surround this diagnosis. It highlights the difficulties clinicians face in providing clear answers in the absence of established knowledge, while also showing that patient concerns are often highly individual. These concerns may vary depending on factors such as age, sex, and personal circumstances, and therefore call for a tailored and patient-centered approach to care.

Characterization of the BDUC population

Heterogeneity and female predominance

The BDUC patient population is a heterogeneous group, presumably consisting of different subpopulations. Despite the many variabilities, some basic characteristics can be assigned to the BDUC population. It is highly remarkable that the majority of the BDUC patients are female, with several cohort studies reporting percentages ranging from 59% to 91% of the study populations [4-6, 13]. This could be explained by women-specific hemostatic challenges including menstruation and childbirth. Heavy menstrual bleeding (HMB) is a common symptom in BDUC patients and patients with other (mild) bleeding disorders [6, 13-15]. When looking at populations of women with HMB, 39% of adolescents and 16% of adults turn out to have a bleeding disorder as the underlying cause [16]. It is also known that HMB is an important contributor to iron deficiency and iron deficiency anemia, which has an additional negative impact on quality of life [17]. HMB is also a symptom that is often underreported as many patients consider it normal, especially in families where several generations have suffered from HMB [18]. Not only normalization of the symptoms but also the stigmatization that still surrounds the subject of menstruation leads to underreporting [18]. While increasing awareness of HMB is crucial, it should be accompanied by attention to other bleeding symptoms as well. In clinical practice, once HMB is recognized, there is often a tendency to overlook or not inquire about additional bleeding manifestations, which may lead to an incomplete assessment of the patient's overall bleeding phenotype. In conclusion, caregivers should pay greater attention to this frequently underestimated symptom and its broader impact on patients' daily lives, both in BDUC and beyond. Despite the female predominance, female BDUC patients are diagnosed at a later age than men. In general, the age at diagnosis in BDUC and other mild bleeding disorders is higher because patients present at a later age when more hemostatic challenges have been faced [13]. Although there often is no clear evidence for heredity, 30-40% of BDUC patients report a positive family history [4, 5].

Bleeding phenotype

Besides the women-specific bleeding symptoms, other typical bleeding symptoms reported by BDUC patients are mucocutaneous bleeding including epistaxis, hematomas and gingival bleeding, but also bleeding during or after surgical or dental interventions [5, 6, 13, 14, 19]. The bleeding phenotype resembles that of patients with an established (mild) bleeding disorder such as von Willebrand disease or platelet function disorders [5]. Based on bleeding phenotype alone, it is impossible to distinguish between BDUC and patients with other mild bleeding disorders [20].

Knowledge gaps in BDUC

The most important knowledge gaps regarding BDUC that were identified in **chapter 2** and will be further discussed in the remainder of this chapter were the BDUC definition,

the diagnostic process, the underlying pathophysiological mechanism (which will be discussed in part II of this chapter) and management and treatment. Other important knowledge gaps concerning patient reported outcomes, care pathways and patient journeys will be included in future BDUC studies.

The definition of BDUC

The first step in an attempt to standardize the heterogeneous group of BDUC patients was to establish a clear definition. Definitions used to describe BDUC were very heterogeneous, but there were two recurrent elements in almost all of them: (a) the presence of an increased bleeding tendency and (b) the absence of abnormal laboratory test results [5, 13, 21]. One of the main goals of the National BDUC survey, described in **Chapter 3**, was to define BDUC in the Netherlands. Based on our survey, we propose the following definition for BDUC: patients with an increased bleeding tendency, based on an elevated (ISTH)-BAT score (≥6 in women and ≥4 in men) or clinical gestalt, in whom no abnormalities are found in a specified panel of laboratory tests and in whom known bleeding disorders are excluded and other causes for a bleeding tendency are considered. This definition aligns with the BDUC definition published by the ISTH SSC in 2024 [15].

The importance of a clear BDUC definition lies not only in the effort to standardize this heterogeneous patient group, but also in enabling the establishment of a clinical diagnosis despite the absence of a known causal mechanism. Assigning a BDUC diagnosis provides reassurance to patients that a comprehensive diagnostic evaluation has been completed and that all known bleeding disorders and other potential causes have been excluded. Furthermore, it allows clinicians to inform patients about available care within hemophilia treatment centers, warning symptoms, situations in which medical attention should be sought, lifestyle factors and (herbal) medications affecting hemostasis, as well as issues related to heredity and genetic counseling. Importantly, a well-defined diagnostic framework also facilitates the development of a personalized treatment plan, even in the absence of a clearly identified pathophysiological mechanism, thereby improving continuity and quality of care for BDUC patients [22, 23].

Current practice in the Netherlands and beyond - Multidisciplinary diagnostic approach

In our center, all patients undergoing further evaluation for an increased bleeding tendency are discussed in a multidisciplinary meeting, where experts from different specialties jointly review the clinical and laboratory findings. Based on this discussion, a decision is made on whether additional investigations are required or whether a diagnosis can be established. For BDUC patients, this process can be lengthy, depending on whether diagnostic tests are performed sequentially or all at once [24]. Establishing a BDUC diagnosis is not always straightforward. Determining clinically relevant bleeding is

challenging: the ISTH-BAT has well-recognized limitations, and clinical gestalt is highly dependent on the physician's experience [20]. Once a BDUC diagnosis is made, new challenges arise regarding the need for a preventative treatment plan. The absence of clear guidelines, particularly regarding treatment and management, contributes to heterogeneity in the management of BDUC patients, both between centers and among individual specialists, which may adversely affect the quality of care provided.

The ISTH SSC has published a communication document outlining the minimal diagnostic steps required to establish a BDUC diagnosis, as well as providing initial guidance on patient management [15]. With regard to the diagnostic pathway, results from the national survey demonstrated that the laboratory tests performed in Dutch hemophilia treatment centers (HTCs) were largely consistent with those recommended by the ISTH SSC (**chapter 3**). An international ISTH SSC survey reported that platelet function tests were performed less frequently than in Dutch HTC's [25]. This discrepancy may be explained by the limited availability of platelet function assays, which are costly, time-consuming and require highly specialized personnel. Additionally, in most peripheral hospitals, several of the laboratory assays required for establishing a BDUC diagnosis are not readily available. As a result, many patients with an increased bleeding tendency from across the country are referred to hemophilia treatment centers, including our own, for specialized hemostasis testing. This raises an important question: can a diagnosis of BDUC be made if not all of the "required" assays have been performed due to limited availability? At the same time, it is worth considering whether it is truly necessary or desirable for all such patients to be referred to specialized centers.

Patient registration and awareness

Following a BDUC diagnosis, new challenges arise concerning patient management, treatment and follow-up. Approximately 20% of respondents in the national survey reported that BDUC patients were not registered in Dutch HTCs, meaning that these patients did not receive a formal diagnosis. This lack of registration may contribute to both loss to follow-up and underestimation of the true prevalence of BDUC. In addition, these patients are often not provided with an individualized treatment plan. Several factors may underlie this issue. On the one hand, limited awareness of BDUC may result in less attention being paid to these patients. On the other hand, some physicians remain skeptical about the diagnosis itself, regarding the absence of abnormal laboratory findings as evidence that "nothing is wrong." Such skepticism may be reinforced by the difficulties encountered in correctly assessing heavy menstrual bleeding complaints in female patients. In case where patients are registered, follow-up practices appear to depend largely on the severity of the bleeding phenotype and are typically limited to periods of active bleeding or hemostatic challenges such as surgery or delivery.

Current treatment practices

Treatment strategies for BDUC are not evidence-based, as no clinical trials have yet evaluated the efficacy or safety of (prophylactic) interventions. Current practice is mainly experience-based, sometimes relying on limited available literature. (Prophylactic) treatment is highly individualized and dependent on the type of surgical intervention [12, 13, 15, 19, 26]. Nationally and internationally, tranexamic acid and desmopressin are the most frequently used treatment modalities for BDUC patients [15, 25]. Clearly, significant knowledge gaps remain, particularly regarding treatment efficacy, prevention of bleeding complications, safety and the potential thrombotic risk.

Therapeutic dilemmas

Consider a BDUC patient scheduled for knee replacement surgery. The patient has experienced postoperative bleeding complications following previous procedures, thus a preventive treatment plan is required. The most commonly available options are tranexamic acid, desmopressin, and platelet transfusion. However, in this case the patient presents with markedly elevated VWF levels (>150%), raising the question whether desmopressin can be safely administered. What if such treatment induces thrombosis? Alternatively, should one resort to platelet transfusion, despite the associated risk of HLA alloimmunization, which could pose significant challenges in the event of future (potentially life-threatening) bleeding episodes? These examples illustrate the therapeutic dilemmas faced by physicians in the management of BDUC patients. Now consider the same patient requiring only minor surgery, such as the excision of a nevus. In this scenario, a platelet transfusion would likely be disproportionate, and tranexamic acid alone might suffice. This further underscores that the choice of treatment is highly dependent on the clinical context, and that a 'one-size-fits-all' approach is not applicable in the management of BDUC patients. In this regard, the development of a practical framework or guidance document outlining treatment options for different clinical scenarios would already be highly valuable in supporting clinicians in their decision-making. A nicely illustrated example of such a guidance document was published previously by Baker et al., emphasizing the need for a personalized approach in the treatment strategy [12].

FUTURE PERSPECTIVES PART I: TOWARDS STANDARDIZED DEFINITIONS, EVIDENCE-BASED MANAGEMENT, AND IMPROVED PATIENT EXPERIENCE

In part one of this thesis, a consensus BDUC definition was reached based on results from a national BDUC survey that was conducted by hemostasis experts from all Dutch hemophilia treatment centers (**chapter 3**). This definition was in line with the one that, at the time, had been published shortly beforehand by the ISTH SSC. Not only will a clear

BDUC definition contribute to a more uniform clinical approach, the process leading to this definition has also raised awareness among health care professionals, which may in turn reduce loss to follow-up in the future. One of the aims of the survey was to determine which diagnostic hemostasis tests should be performed prior to a BDUC diagnosis. The consensus recommendations provide guidance for the diagnostic work-up of BDUC patients and are intended to avoid excessive testing. Current clinical practice regarding treatment and follow-up was also reported in the survey. These aspects of BDUC require future research, as current recommendations lack strong evidence-based support. A first, pragmatic approach would likely involve a prospective cohort study documenting the (prophylactic) treatments administered and evaluating their association with peri-procedural bleeding complications. However, a randomized controlled trial (RCT) on prophylactic treatment in BDUC patients would be required to really assess the efficacy and safety of currently used treatment modalities, including antifibrinolytic agents and desmopressin.
The BDUC-iN study is a prospective multicenter cohort study that has recently started, investigating a variety of BDUC knowledge gaps in different work packages, including advanced hemostasis testing for undiagnosed bleeding disorders and platelet function disorders, application of rich full blood count data for BDUC, Multi-OMICS, endothelial models, health related quality of life, quality of care and care pathways. Research into treatment modalities will be incorporated in a national multi-center BDUC in the Netherlands (BDUC-iN) study, which will contribute to stronger evidence-based advice regarding this topic.

Two knowledge gaps that were identified in **chapter 2** of this thesis, but were not comprehensively discussed, are the quality of life and patient journey of BDUC patients. These are important, yet often overlooked aspects of patient care that deserve attention in future studies, as it already has been shown that bleeding symptoms, including HMB in women in particular, can pose a high burden on daily life activities [27]. Receiving a BDUC diagnosis may provide patients with a sense of being acknowledged and reassured that alternative causes of their bleeding tendency have been thoroughly excluded. Moreover, registration as BDUC allows for support by medical experts, ensuring that patients know where to seek help when needed. On the other hand, there is also a risk of over diagnosis as well as both over- and under treatment, and many uncertainties regarding the condition inevitably remain. For treating physicians, establishing a diagnosis may provide a certain degree of guidance. However, it often leaves a sense of dissatisfaction, by the lack of a pathophysiological explanation for their bleeding tendency. To further investigate quality of life and care, a diverse group of BDUC patients from the national BDUC-iN study will be selected to explore patient experiences through online interviews and focus groups. Topics to be addressed include experiences with current care, satisfaction with the

diagnostic pathway, treatment, accessibility of care, and unmet needs. In addition to patients, health care providers will also be interviewed.

PART II: PIECING IT TOGETHER: FUNCTIONAL TESTING IN THE DIAGNOSTIC WORK-UP OF BDUC

Advanced assessment of hemostasis

To further unravel the underlying bleeding mechanisms in BDUC, we investigated different phases of hemostasis using a variety of advanced (global) assays. These assays included platelet function tests and a flow chamber model to assess primary hemostasis, thrombin generation as a marker of secondary hemostasis, and viscoelastic testing to evaluate fibrinolysis. By applying these complementary approaches, we aimed to obtain a more integrated picture of the hemostatic balance in this heterogeneous group of patients.

Primary hemostasis

In **chapter 7**, within the TiN study, we confirmed that light transmission aggregometry (LTA) remains the reference method for platelet function testing. However, since LTA may fail to show mild platelet function disorders and storage pool disease, additional assays such as flow cytometry-based platelet activation (PACT) and secretion tests were able to detect abnormalities in patients with normal LTA results, thereby modestly increasing the diagnostic yield [28-30]. In **chapter 8**, the Maastricht flow chamber, a multiparameter microfluidic model that incorporates whole blood and physiological shear conditions, provided additional insights by identifying shear-dependent platelet function defects in more than half of the BDUC patients studied. This finding was further illustrated in the individual case presented earlier in this chapter: the young woman with a significant bleeding history and a BDUC diagnosis also underwent flow chamber analysis, which revealed flow-related platelet dysfunction. These results demonstrate how advanced assays can uncover defects not captured by conventional testing, and may help to explain otherwise unexplained bleeding symptoms.

Secondary hemostasis

With respect to secondary hemostasis, the thrombin generation assay (TGA) revealed abnormalities consistent with a bleeding tendency in about one quarter of BDUC patients in **chapter 6**. This is in line with a part of the previous studies that have investigated TGA in BDUC [13, 21, 31-34]. However, *despite efforts to increase the sensitivity of the assay by adapting the reagents,* no clear difference in the apparent ability of the three reagents to identify a bleeding tendency in this study population could be detected. Since abnormal TGA results cannot precisely localize

the hemostatic defect, the assay's diagnostic value in this population appears limited, serving mainly to suggest a possible direction for further investigation.

Fibrinolysis

Finally, fibrinolysis was assessed using the tPA-ROTEM assay, which induced fibrinolysis in whole blood by adding tissue plasminogen activator (tPA) in **chapter 5** [35]. A hyperfibrinolytic profile was detected in approximately 20% of BDUC patients, suggesting that excessive fibrinolysis contributes to the bleeding tendency in a subset of individuals. This is consistent with previous reports, although the absence of a universally accepted, standardized fibrinolysis assay continues to limit broader implementation in clinical practice [32, 36, 37].

Taken together, these studies highlight that no single test sufficiently explains the bleeding phenotype in BDUC. Nonetheless, combining different assay modalities across the spectrum of hemostasis, ranging from platelet function under flow to thrombin generation and fibrinolysis, can help uncover subtle abnormalities which may guide future diagnostic strategies and provide patients with more targeted counseling and management.

FUTURE PERSPECTIVES PART II: INNOVATIVE DIAGNOSTIC APPROACHES IN BDUC

Future research in Bleeding Disorder of Unknown Cause (BDUC) should focus on further elucidating the underlying mechanisms of bleeding by integrating advanced hemostasis assays into multidimensional diagnostic strategies. The findings presented in this thesis provide several hypothesis-generating observations that can guide such developments.
In **Chapters 5**, **6**, and **8**, three different advanced hemostasis assays were applied to BDUC patients from the ProBe-AHP cohort. Although all patients were recruited within the same study, not all underwent the full panel of assays due to plasma availability and assay-specific limitations. Consequently, the number of patients varied per test, with a total of 146 BDUC patients undergoing at least one advanced assay. Among these, the tPA-ROTEM was most frequently performed (n=92), followed by the thrombin generation assay (TGA, n=83) and the Maastricht flow chamber assay (n=47). Only a small subset underwent all three assays (n=12) (Figure 1).

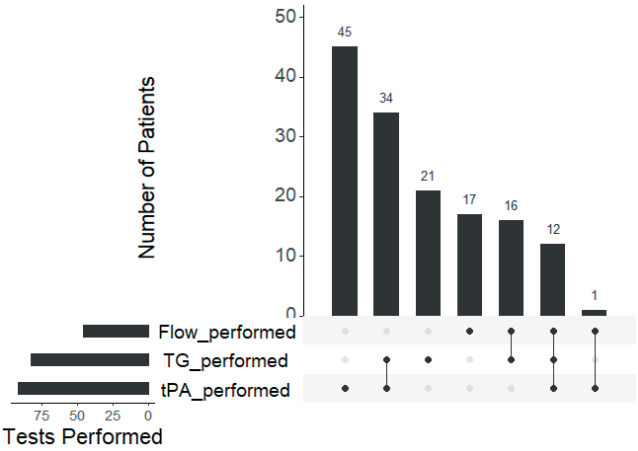


Figure 1: Upset plot illustrating the number of patients who underwent each of the three additional assays: flowchamber assay (flow), thrombin generation assay (TG) and tPA-ROTEM assay (tPA). Horizontal bars on the left represent the total number of patients tested for each assay, regardless of other assays performed. Vertical bars represent the number of patients who underwent the specific combination of assays indicated by the connected dots below the bars.

When exploring the overlap of abnormal results, 39% of patients demonstrated at least one abnormal finding (Figure 2). The distribution of isolated versus combined abnormalities suggests that distinct pathophysiological mechanisms may underlie the BDUC phenotype. For instance, isolated abnormalities in the flow chamber indicate possible shear-dependent platelet dysfunction, whereas tPA-ROTEM abnormalities may point toward fibrinolytic imbalance. The limited overlap between assays underscores the multifactorial and heterogeneous nature of BDUC, suggesting that no single unifying mechanism accounts for all cases.

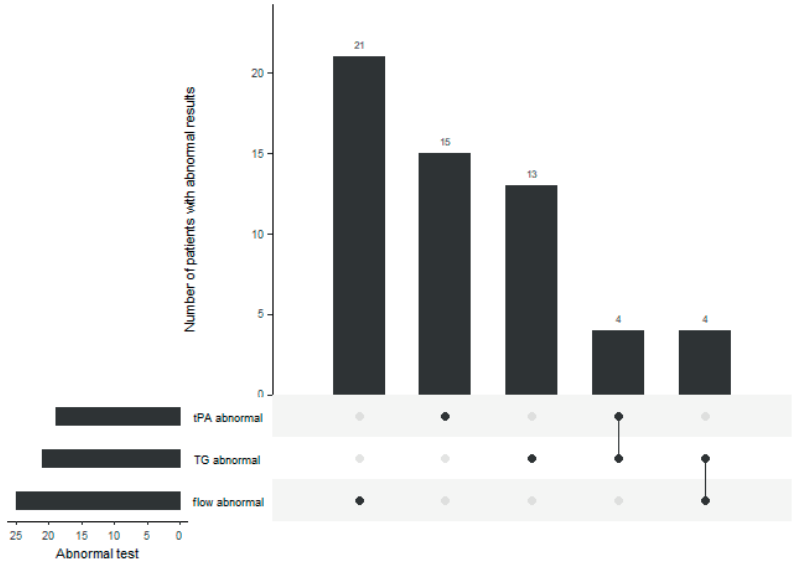


Figure 2: Upset plot showing the distribution and overlap of abnormal results among the three assays. Horizontal bars represent the total number of patients with an abnormal result in each assay, independent of abnormalities in other assays. Vertical bars represent the number of patients with the specific combination of abnormal results indicated by the connected dots below. The largest proportion of abnormalities was observed in flow chamber alone (n=21), followed by isolated tPA-ROTEM (n=15) and TG (n=13) abnormalities.

Although these findings were exploratory and based on unequal test distribution, they provide a conceptual framework for future research. Specifically, they support the idea that advanced hemostasis assays can help identify biologically distinct subgroups within the BDUC population, each characterized by a different hemostatic imbalance. Future studies should aim to validate these potential subgroups in larger, well-characterized cohorts and investigate whether they correspond to distinct clinical phenotypes or treatment responses.

To further advance our understanding, future research should also focus on mechanistic studies. These could include refinement of existing models, such as modifying the Maastricht flow chamber by incorporating endothelial cells, or the implementation of other flow-based assays, such as the Total Thrombus-formation Analysis System (T-TAS). Flow cytometry based assays (e.g., PACT) may be used to explore specific platelet receptor or activation defects, while advanced von Willebrand factor functional analyses could yield new insights in patients with low-normal VWF levels or prolonged PFA-200 results.

Additionally, (platelet) proteomic approaches could uncover novel proteins involved in hemostatic regulation, especially when performed in an unbiased manner.

Finally, comprehensive phenotyping and genotyping in families with multiple affected members may provide further insight, even though previous studies have demonstrated a limited diagnostic yield of targeted genetic panels [38]. The integration of these advanced diagnostic modalities, combining global and targeted hemostasis testing, will be essential to move from a descriptive toward a mechanistic understanding of BDUC.

The forthcoming prospective, multicenter BDUC-iN study aims to build upon these insights, by investigating the true underlying pathophysiological mechanisms of BDUC and by testing the concept of subgroup stratification based on assay profiles and clinical features. Ultimately, these efforts may pave the way toward more personalized diagnostic and therapeutic approaches for patients with BDUC.

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ADDENDUM

IMPACT PARAGRAPH

ENGLISH SUMMARY

NEDERLANDSE SAMENVATTING

LIST OF PUBLICATIONS

CURRICULUM VITAE

DANKWOORD

IMPACT PARAGRAPH

This chapter discusses the valorization of the research conducted in this thesis. It highlights the clinical and societal relevance of findings, identifies the populations that may benefit from them, considers their innovative aspects and outlines potential directions for future application and development.

Research field and main objectives

Bleeding disorder of unknown cause (BDUC) is increasingly recognized as a distinct clinical entity. Patients typically present with bleeding symptoms similar to those of patients with mild bleeding disorders such as von Willebrand disease or platelet function defects, yet standard laboratory evaluation fails to identify an underlying abnormality [1]. BDUC accounts for a large proportion of the patients with an increased bleeding tendency that are referred to hemostasis experts, estimates range from 40–70%, and is associated with substantial morbidity [2]. Although life-threatening bleeding is uncommon, recurrent symptoms such as heavy menstrual bleeding, epistaxis, and hematomas substantially affect quality of life [3]. In addition, bleeding complications following medical procedures or childbirth often necessitate interventions, sometimes including transfusions and surgical re-exploration [4-7]. Women are disproportionately affected, largely due to gynecological and obstetric bleeding manifestations [4, 8]. Despite the clinical burden, treatment approaches remain empirical and diagnostic strategies inconsistent, reflecting major knowledge gaps regarding definition, underlying bleeding mechanisms, and management [5, 6, 8].

The studies described in this thesis were therefore designed to better characterize BDUC and to explore potential underlying mechanisms using advanced hemostasis assays. The findings are relevant not only from a scientific perspective but also from a societal and clinical standpoint. Specifically, this work has contributed to the formulation of a national BDUC definition, increasing awareness of this patient population in Dutch hemophilia treatment centers, and has provided novel insights into possible pathophysiological mechanisms by applying advanced platelet function tests, a thrombin generation assay, a global fibrinolytic assay (tPA-ROTEM) and a flow model (Maastricht flow chamber).

Relevance and utilization of the key findings

This thesis presents results that are relevant to the characterization of BDUC on the one hand, and to the investigation of the underlying pathophysiological bleeding mechanism on the other.

Characterization of BDUC and formulation of BDUC definition

A key contribution of this thesis has been the development of a national BDUC definition, formulated in collaboration with all Dutch Hemophilia Treatment Centers (HTCs) and aligned with the ISTH SSC criteria that were published in 2024. Standardization of terminology and diagnostic criteria is more than a scientific exercise: it offers practical benefits for both patients and clinicians.

For patients, the definition validates their symptoms by confirming that a thorough work-up has excluded known bleeding disorders and other causes for bleeding. This recognition not only reassures patients but also facilitates their registration in specialized treatment centers, where they can receive structured care and guidance in high-risk situations such as surgery or childbirth. For clinicians, the definition offers a framework that reduces unnecessary or repetitive testing, guides diagnostic decisions, and facilitates systematic follow-up. Beyond standardization, the definition lays the groundwork for more uniform diagnostic and research strategies. It reduces unnecessary or repetitive testing, improves awareness among healthcare professionals and facilitates systematic follow-up. In the long term, this foundation enables research into optimized treatment approaches, patient-reported outcomes and care pathways. Ultimately, the work contributes to more consistent clinical care and it functions as a framework for advancing knowledge in BDUC.

Investigation of underlying bleeding mechanisms using advanced hemostatic assays

Routine hemostasis assays often fail to explain the bleeding tendency in BDUC, partly because they are performed under static conditions and fail to capture the complexity of in vivo hemostasis. This thesis therefore applied several advanced hemostasis assays. The tPA-ROTEM revealed hyperfibrinolytic profiles in a subset of patients, suggesting that dysregulated fibrinolysis may contribute to bleeding in BDUC and underscoring the potential value of antifibrinolytic therapy. The thrombin generation assay identified abnormalities in approximately one quarter of patients, though its diagnostic value remains limited given the variability in test modalities and the absence of clear mechanistic insights. Additional platelet function assays modestly improved diagnostic yield, while the Maastricht flow chamber detected shear-dependent platelet dysfunction in over half of patients studied, highlighting the importance of assessing hemostasis under flow. Despite these advances, major questions remain. Future models should incorporate vascular wall elements such as endothelial cells, which directly interact with platelets and plasma proteins and are likely to influence bleeding mechanisms in vivo. Complementary approaches, including platelet proteomics, electron microscopy, advanced von Willebrand diagnostics, and genetic testing, offer further opportunities to unravel the pathophysiological mechanisms of BDUC. Integration of these tools into clinical research could ultimately enable more precise patient stratification and personalized treatment strategies.

Knowledge dissemination to target groups

Although primarily scientific in nature, the findings of this thesis have important clinical and societal implications, particularly for women with heavy menstrual bleeding (HMB). HMB is the most common presenting symptom of BDUC and has profound effects on physical health, psychosocial well-being, and socioeconomic participation. By improving recognition of BDUC and outlining diagnostic strategies, this work contributes directly to better management of women with unexplained bleeding. The identification of subgroups, such as those with shear-dependent platelet dysfunction or hyperfibrinolysis, may in the future guide targeted treatment approaches, including antifibrinolytic therapy, and help prevent complications during childbirth. At the healthcare system level, the work highlights the importance of structured referral to specialized centers, where diagnostic clarity, individualized treatment plans, and anticipatory guidance in high-risk situations can be provided. Greater awareness among gynecologists, general practitioners, and other primary care providers is essential, given that they are often the first to encounter women with unexplained HMB. From a broader societal perspective, this thesis contributes to reducing uncertainty for both patients and healthcare providers, improving health-related quality of life, and laying the groundwork for future guideline development. Dissemination of results through peer-reviewed publications, conference presentations, and engagement with patient organizations ensures that the insights gained reach diverse audiences, from fundamental researchers to clinicians and patient communities. Looking forward, the findings of this thesis establish a foundation for national and international collaboration in BDUC research. The standardized definition facilitates consistent patient inclusion, while advanced assays provide new approaches to explore pathophysiological mechanisms. Together, these steps bring us closer to the development of evidence-based guidelines, optimized diagnostic pathways, and tailored treatment strategies. Ultimately, this work aims to enhance both clinical practice and patient experience, improving outcomes for individuals with bleeding disorder of unknown cause.

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ENGLISH SUMMARY

The majority of individuals who present to a hemostasis specialist with clinically relevant bleeding tendencies do not receive a definitive diagnosis of a coagulation disorder following comprehensive clinical and laboratory evaluation. In such cases, the condition is referred to as a bleeding disorder of unknown cause (BDUC). Two main criteria must be met to establish a diagnosis of BDUC: (1) the presence of a clinically significant bleeding tendency, determined either by an elevated bleeding score and/or the clinician's judgment, and (2) the absence of abnormalities in a standard panel of laboratory tests. Additionally, it is essential to exclude alternative causes of increased bleeding, such as thyroid or liver disorders. These criteria result in a heterogeneous patient population, with bleeding phenotypes ranging from mild to severe. The clinical presentation of BDUC strongly overlaps with that of other mild bleeding disorders, such as von Willebrand disease and mild platelet function disorders. The lack of an identifiable cause contributes to uncertainty for both patients and clinicians, particularly because evidence-based guidelines for management and follow-up are lacking. Current diagnostic methods are insufficient, highlighting the need for more advanced laboratory techniques.

The aim of this thesis was twofold: first, to characterize the heterogeneous BDUC population (Part I), and second, to explore potential underlying mechanisms that may explain the bleeding tendency in these patients using advanced diagnostic approaches (Part II).

Chapter 1 provides an overview of the theoretical background of hemostasis, which forms the context for this thesis. The diagnostic pathway followed by patients presenting with increased bleeding tendencies is described, which for the majority of patients results in a BDUC diagnosis. Furthermore, advanced laboratory techniques with potential added value for this patient group are discussed. Based on this background, specific objectives were formulated and addressed in the subsequent chapters.

Chapter 2 presents an illustrated review of current knowledge and knowledge gaps regarding BDUC. Known patient characteristics are summarized, along with the capabilities and limitations of bleeding assessment tools and other instruments used to quantify bleeding tendency. The major knowledge gaps identified relate to the definition of BDUC, underlying bleeding mechanisms, diagnostic processes, treatment and follow-up, patient-reported outcomes, and care pathways. For each of these domains, the available literature is summarized and visually presented, followed by recommendations for future research.

Chapter 3 addresses some of the knowledge gaps identified in Chapter 2. Given the relatively recent introduction of the BDUC concept, considerable variation exists in the criteria applied for diagnosis, investigation, and management, both in literature and in clinical practice. A national survey among hemostasis experts from all Dutch hemophilia treatment centers was conducted to map current clinical practice. This was complemented by an international survey, allowing a comparison between Dutch practice and international recommendations. The results indicate that Dutch practice largely aligns with international guidance but also reveals some differences. The national findings may complement international recommendations and together form a basis for the development of uniform guidelines for BDUC care.

Chapter 4 describes a scoping review on the clinical applicability of the Total Thrombus-formation Analysis System (T-TAS), a laboratory assay that simulates blood flow conditions. As this test may be relevant for BDUC, a review was conducted of studies applying T-TAS in various clinical contexts, including bleeding disorders. The results show that T-TAS can detect severe forms of von Willebrand disease and platelet function disorders, but it is insufficiently sensitive for milder forms. The potential value of this test in BDUC therefore requires further investigation.

Chapter 5 reports the application of the tPA-ROTEM assay in BDUC patients. This viscoelastic method measures the resistance generated during clot formation. Addition of tissue plasminogen activator (tPA) initiates fibrinolysis. Results demonstrated that a subset of BDUC patients exhibits hyperfibrinolysis, providing a plausible explanation for their bleeding tendency. These findings also support the rationale for the use of tranexamic acid, an antifibrinolytic drug widely applied in this patient group.

Chapter 6 investigates the diagnostic value of the thrombin generation (TG) assay in BDUC. TG measures thrombin, which is ultimately converted to fibrin, the final product of secondary hemostasis. Although various reagents were used to assess sensitivity for subtle abnormalities, results were not convincingly indicative. Approximately one quarter of patients displayed one or more abnormalities; however, because this global hemostasis assay primarily reflects the end of the coagulation cascade, mild defects in earlier steps may be masked, and the precise cause remains unclear. The added diagnostic value of TG in BDUC thus appears limited.

Chapter 7 focuses on the diagnostic value of multiple platelet function assays in patients suspected of having a platelet function disorder, excluding von Willebrand disease. Four platelet function tests were applied: (1) light transmission aggregometry (LTA), which measures platelet aggregation in response to different agonists; (2) platelet ADP content, which assesses ADP and ATP secretion from platelet granules as a measure of activation;

(3) flow cytometric analysis of platelet receptor expression; and (4) the flow cytometry based platelet activation test (PACT), which evaluates platelet activation in response to various agonists. Results showed that LTA, the current gold standard, yielded most diagnoses. However, more specialized methods such as ADP content measurement and flow cytometry detected additional abnormalities in some patients with strong clinical suspicion not identified by LTA.

Chapter 8 presents results from flow chamber experiments conducted in 47 BDUC patients. This assay more closely simulates in vivo conditions than conventional tests by using whole blood under controlled flow. Microscopic images of the flowing blood were recorded and subsequently analyzed. Abnormalities indicative of underlying platelet function defects, which went undetected by conventional techniques, were observed in more than half of the patients.

Chapter 9 presents a case study of a family with the rare fibrinogen disorder hypodysfibrinogenemia, in which a novel genetic mutation was identified and confirmed with proteomics. Although these patients may exhibit both bleeding and thrombotic tendencies, standard laboratory tests primarily revealed a bleeding phenotype. Flow chamber experiments and TG additionally demonstrated a prothrombotic tendency, which ultimately informed adjustments to the treatment plan.

Finally, **Chapter 10** summarizes the main findings of this thesis in a general discussion and provides recommendations for future research. Application of advanced hemostasis assays in BDUC patients has generated new insights and indications of underlying bleeding mechanisms. However, larger-scale studies are required to confirm these findings and further elucidate their pathophysiological basis.

NEDERLANDSE SAMENVATTING

De meerderheid van de personen die zich met een klinisch relevante bloedingsneiging presenteert bij een hemostase-expert, ontvangt na uitgebreid klinisch en laboratoriumonderzoek geen definitieve diagnose van een stollingsstoornis. In dergelijke gevallen wordt er gesproken van een *onverklaarde bloedingsneiging*, ook wel *Bleeding Disorder of Unknown Cause* (BDUC) genoemd. Voor het stellen van de diagnose BDUC moeten twee belangrijke criteria worden vervuld: (1) er is sprake van een klinisch relevante bloedingsneiging, vastgesteld op basis van een verhoogde bloedingsscore en/of het klinisch oordeel van de arts, en (2) er worden geen afwijkingen aangetoond in een standaardpakket van laboratoriumtesten. Daarnaast is het essentieel om alternatieve oorzaken van een verhoogde bloedingsneiging, zoals schildklier- of leveraandoeningen, uit te sluiten. Deze criteria resulteren in een heterogene patiëntengroep met bloedingsfenotypen die variëren van mild tot ernstig. De klinische presentatie van BDUC overlapt sterk met die van andere milde bloedingsstoornissen, zoals de ziekte van von Willebrand en milde plaatjesfunctiestoornissen. Het ontbreken van een aantoonbare oorzaak leidt tot onzekerheid bij zowel patiënten als behandelaars, mede doordat richtlijnen voor behandeling en follow-up ontbreken. De huidige diagnostische methoden blijken ontoereikend, hetgeen de noodzaak onderstreept van meer geavanceerde laboratoriumtechnieken.

Het doel van dit proefschrift was tweeledig: enerzijds het karakteriseren van de heterogene BDUC-populatie (deel I), en anderzijds het exploreren van mogelijke onderliggende mechanismen die de bloedingsneiging bij deze patiënten kunnen verklaren, met behulp van geavanceerde diagnostische technieken (deel II).

Hoofdstuk 1 biedt een overzicht van de theoretische achtergrond van het hemostaseproces, relevant voor de verdere context van dit proefschrift. Tevens wordt het diagnostisch traject beschreven dat patiënten met een verhoogde bloedingsneiging doorlopen, wat voor het merendeel van de patiënten resulteert in een BDUC diagnose. Daarnaast wordt ingegaan op geavanceerde laboratoriumtechnieken die potentieel meerwaarde kunnen hebben voor deze patiëntengroep. Op basis hiervan zijn specifieke doelstellingen geformuleerd die in de daaropvolgende hoofdstukken worden uitgewerkt.

Hoofdstuk 2 presenteert een geïllustreerde review van de huidige kennis en kennishiaten rondom BDUC. Bekende patiëntkenmerken worden overzichtelijk weergegeven, evenals de mogelijkheden en beperkingen van bloedingsscores en andere instrumenten om bloedingsneiging te kwantificeren. De belangrijkste kennishiaten die werden geïdentificeerd betreffen: de definitie van BDUC, het onderliggende bloedingsmechanisme, het diagnostisch proces, behandeling en follow-up, patiënt gerapporteerde uitkomsten

en zorgpaden. Voor elk van deze domeinen wordt de beschikbare literatuur samengevat en visueel gepresenteerd, waarna aanbevelingen voor toekomstig onderzoek worden geformuleerd.

Hoofdstuk 3 verdiept zich in enkele van de in hoofdstuk 2 geïdentificeerde kennishiaten. Gezien de relatief recente introductie van BDUC bestaat er aanzienlijke variatie in de gehanteerde criteria voor de definitie, diagnostiek en behandeling, zowel in de literatuur als de klinische praktijk. Via een nationale enquête onder hemostase-experts uit alle Nederlandse hemofiliebehandelcentra werd de huidige klinische praktijk geïnventariseerd. Parallel werd een internationale enquête uitgevoerd, waardoor een vergelijking mogelijk was tussen de Nederlandse situatie en internationale aanbevelingen. De resultaten tonen aan dat de Nederlandse praktijk grotendeels overeenkomt met internationale adviezen, maar ook enkele verschillen vertoont. De nationale bevindingen kunnen als aanvulling dienen op de internationale adviezen en gezamenlijk een fundament vormen voor de ontwikkeling van eenduidige richtlijnen voor de zorg rondom BDUC.

Hoofdstuk 4 beschrijft een *scoping review* naar de klinische toepasbaarheid van het *Total Thrombus-formation Analysis System* (T-TAS), een laboratoriumtest die zich onderscheidt door het gebruik van bloedstroomcondities. Aangezien deze test mogelijk relevant is voor BDUC, werd een overzicht opgesteld van studies waarin T-TAS werd toegepast in diverse klinische contexten, waaronder bloedingsstoornissen. De resultaten laten zien dat T-TAS ernstige vormen van de ziekte von Willebrand en plaatjesfunctiestoornissen kan detecteren, maar onvoldoende gevoelig blijkt voor mildere vormen. De potentiële waarde van deze test bij BDUC dient derhalve nader onderzocht te worden.

Hoofdstuk 5 rapporteert de toepassing van de tPA-ROTEM test bij BDUC-patiënten. Deze visco-elastische methode meet de weerstand veroorzaakt door stolselvorming. Aan deze test wordt tissue plasminogeen activator (tPA) toegevoegd, wat leidt tot de initiatie van fibrinolyse. De resultaten tonen dat een subgroep van BDUC-patiënten hyperfibrinolyse vertoont, wat een plausibele verklaring kan zijn voor hun bloedingsneiging. Deze bevinding ondersteunt tevens de rationale voor het gebruik van tranexaminezuur, een antifibrinolyticum dat reeds veelvuldig wordt toegepast in deze patiëntengroep.

Hoofdstuk 6 onderzoekt de diagnostische waarde van de trombine generatie test (TG) bij BDUC. TG meet trombine, wat uiteindelijk omgezet wordt in fibrine, het eindproduct van de secundaire hemostase. Hoewel met diverse reagentia werd geëvalueerd of de test gevoeligheid vertoont voor subtiele afwijkingen, bleek dit niet overtuigend het geval. Bij ongeveer een kwart van de patiënten werden wel een of meerdere afwijkingen gevonden, maar aangezien met deze globale hemostase test vooral gekeken wordt naar het einde van de bloedstollingscascade, worden milde afwijkingen in de voorafgaande stappen van

de bloedstolling mogelijks gemaskeerd en blijft de exacte oorzaak van de afwijkingen vooralsnog onbekend. De toegevoegde diagnostische waarde van TG bij BDUC lijkt dan ook eerder beperkt.

Hoofdstuk 7 richt zich op de diagnostische waarde van verschillende plaatjesfunctietesten bij patiënten met een vermoeden van een plaatjesfunctiestoornis, bij wie de ziekte van von Willebrand was uitgesloten. Vier plaatjesfunctietesten werden toegepast: (1) de light transmission aggregometry test (LTA) die de aggregatie van de bloedplaatjes meet in respons op verschillende agonisten, (2) de platelet ADP content test die de secretie meet van ADP en ATP uit de granules van de bloedplaatjes, wat een maat is voor activatie, (3) flowcytometrische analyse van plaatjesreceptor expressie en tot slot (4) flowcytometrische plaatjesactivatie test (PACT) die de activatie van de plaatjes in respons op verschillende agonisten onderzoekt. De resultaten tonen aan dat LTA, de huidige gouden standaard, de meeste diagnoses oplevert. Meer gespecialiseerde methoden, zoals ADP content en flow cytometrie, konden echter aanvullende afwijkingen detecteren bij sommige patiënten met een sterke klinische verdenking die door LTA niet werden geïdentificeerd.

Hoofdstuk 8 beschrijft de resultaten van *flowkamer* experimenten bij 47 BDUC-patiënten. Deze test simuleert de in vivo situatie nauwkeuriger dan conventionele testen door gebruik van volbloed en gecontroleerde bloedstroomcondities. Tijdens het experiment worden microscopische beeldopnamen verkregen van het stromende bloed, die nadien geanalyseerd worden. Bij meer dan de helft van de patiënten werden afwijkingen gedetecteerd die kunnen wijzen op een onderliggende plaatjesfunctiestoornis, welke met conventionele technieken onopgemerkt bleven.

Hoofdstuk 9 presenteert een casus van een familie met de zeldzame fibrinogeenafwijking hypodysfibrinogenemie, waarin een nieuwe genetische mutatie werd geïdentificeerd en bevestigd via proteomics. Hoewel deze patiënten zowel een verhoogde bloedings- als trombosneiging kunnen vertonen, lieten standaardtesten voornamelijk een bloedingsprofiel zien. Flowkamerexperimenten en TG toonden echter ook een trombosneiging aan, wat uiteindelijk heeft geleidt tot aanpassingen in het behandelplan.

Tot slot worden in **hoofdstuk 10** de belangrijkste bevindingen van dit proefschrift samengevat in een algemene discussie en er worden suggesties gedaan voor toekomstig onderzoek. De toepassing van geavanceerde hemostasetesten bij BDUC-patiënten heeft geleid tot nieuwe inzichten en aanwijzingen voor onderliggende bloedingsmechanismen. Grootschaliger onderzoek is echter noodzakelijk om deze bevindingen te bevestigen en de pathofysiologische basis verder te ontrafelen.

LIST OF PUBLICATIONS

Thesis publications

Monard ALL*, Mussert CMA*, van Duijl TT, Kruip MJHA, Henskens YMC, van den Biggelaar M, Schutgens REG, Schols SEM, Fijnvandraat KJ, Meijer K, den Exter PL, Nieuwenhuizen L, van Moort I, Baker RI, O'Donnell JS, Cnossen MH, Heubel-Moenen FCJI. Bleeding disorder of unknown cause: an illustrated review on current practice, knowledge gaps, and future perspectives. Res Pract Thromb Haemost. 2024 Nov 13;8(8):102625.

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*Shared first authorship

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*Shared first authorship

Submitted for publication

Monard ALL, Heubel-Moenen FCJI, Henskens YMC, Schutgens REG, Urbanus RT on behalf of the TiN study group and the BDUC-iN study group. Diagnostic value of platelet function tests, including the flow cytometry based platelet activation test (PACT), in patients with a suspected inherited platelet function disorder. (submitted)

Monard ALL, Moonen L, Hellenbrand D, Verhezen P, Merry I, Derikx F, Lauw M, Beckers EAM, van der Meijden PEJ, Cosemans JMEM, Henskens YMC, Heubel-Moenen FCJI. Microfluidic testing in patients with Bleeding Disorder of Unknown Cause (BDUC). (submitted)

Urlings E, **Monard ALL**, Merry I, Derikx D, van Oerle R, Beckers E, Henskens Y, Spronk H, Heubel-moenen F. Diagnostic value of thrombin generation using different trigger reagents in patients with bleeding disorder of unknown cause (BDUC). (submitted)

Oral presentations

EHA 2025 - Microfluidic testing using the Maastricht flowchamber in patients with Bleeding Disorder of Unknown Cause (BDUC).

DHC 2025 - The use of ATP-release identifies platelet function disorders in patients with bleeding disorder of unknown cause (BDUC) and normal LTA results.

DHC 2024 - Evaluation of innovative laboratory tests to elucidate a thrombotic phenotype in a family with dysfibrinogenemia and a novel *FGG* mutation.

DHC 2024 - Clinical value of laboratory diagnostics for bleeding disorders in general practice.

NVKC voorjaarscongres 2024 - Fibrinolysis assessment with tpa-rotem in patients with bleeding disorders of unknown cause (BDUC).

NVKC voorjaarscongres 2023 - Evaluation of innovative laboratory tests to predict a thrombotic phenotype in a family with dysfibrinogenemia and a novel *FGG* mutation.

PAOKC symposium 2024 - Let it flow: overall stolling analyse met de flowkamer.

Poster presentations

ISTH 2025/EHA 2025/EAHAD 2025 - ATP-Release Identifies Platelet Function Disorders in Patients with Bleeding Disorder of Unknown Cause (BDUC) and Normal LTA Results.

ISTH 2025 - Maastricht flow chamber test in patients with Bleeding Disorder of Unknown Cause (BDUC).

ISTH 2025 - Diagnostic value of platelet function tests in patients with a suspected platelet function defect.

ISTH 2025 - Health related quality of life in patients with bleeding disorder of unknown cause.

ISTH 2025 - Heavy menstrual bleeding impacts quality of life in patients with bleeding disorder of unknown cause**.**

ISTH 2024/EAHAD 2024 - Clinical value of laboratory diagnostics for bleeding disorders in general practice.

ISTH 2024/EAHAD 2024 - Bleeding Disorder of Unknown Cause (BDUC): an illustrated review on current practice, knowledge gaps and future perspectives

ISTH 2024/EAHAD 2024 - The diagnostic value of thrombin generation in patients with bleeding disorders.

ISTH 2024/EAHAD 2024 - Fibrinolysis assessment with tPA-rotem in patients with bleeding disorders of unknown cause (BDUC).

ISTH 2023/EAHAD 2024 - Evaluation of innovative laboratory tests to predict a thrombotic phenotype in a family with dysfibrinogenemia and a novel *FGG* mutation.

CURRICULUM VITAE

Amaury Monard was born on March 27, 1998 in Genk, Belgium. She received her secondary education at the Onze-Lieve-Vrouwe Lyceum in Genk, where she graduated in 2016. With a strong interest in medicine and biomedical sciences, she subsequently enrolled in the Bachelor of Medicine program at Maastricht University. After successfully completing her bachelor's degree in 2019, she continued her medical training in the Master of Medicine at the same university.

During her master's studies, she did both her clinical and scientific participation internships (GEZP and WESP) at the hematology department at the Maastricht University Medical Center + (MUMC+). It was during her scientific internship that she joined the research team led by Prof. Dr. Erik Beckers, Prof. Dr. Yvonne Henskens and Dr. Floor Heubel-Moenen. This first introduction to hematological research marked the beginning of her academic trajectory in the field.

In 2022, after graduating as a medical doctor, she continued her scientific career by starting a PhD trajectory in the same research group. She was appointed as a PhD candidate at the Department of Hematology, Internal Medicine and the Central Diagnostic Laboratory (CDL) at MUMC+, working in close collaboration with the department of Biochemistry, part of the Cardiovascular Research Institute Maastricht (CARIM), Maastricht University.

Her doctoral research focusses on improving diagnostics in bleeding disorders, with a particular emphasis on bleeding disorder of unknown cause (BDUC). This research is embedded in a nationwide collaborative effort, involving representatives of all Dutch hemophilia treatment centers, the SYMPHONY consortium and Sanquin. Together, these partners form the Bleeding disorder of unknown cause in the Netherlands (BDUC-iN) steering group, under the coordination of Dr. Floor Heubel-Moenen.

Within this collaborative framework, her work has centered on advancing the understanding of BDUC and refining diagnostic strategies, with the ultimate goal of improving patient care. The outcomes of this PhD trajectory are presented in the current thesis.

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