Diagnosis And Management Of Inherited And Acquired Bleeding Disorders – Focus On Female Specific Health Issues In Haemostasis And Thrombosis

Caroline Suzanne Barbara Veen

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Diagnostiek en behandeling van aangeboren en verworven stollingsstoornissen – focus op vrouwspecifieke gezondheidsproblemen gerelateerd aan de bloedstolling

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CHAPTER 1

General introduction and outline of thesis

GENERAL INTRODUCTION

The haemostatic system

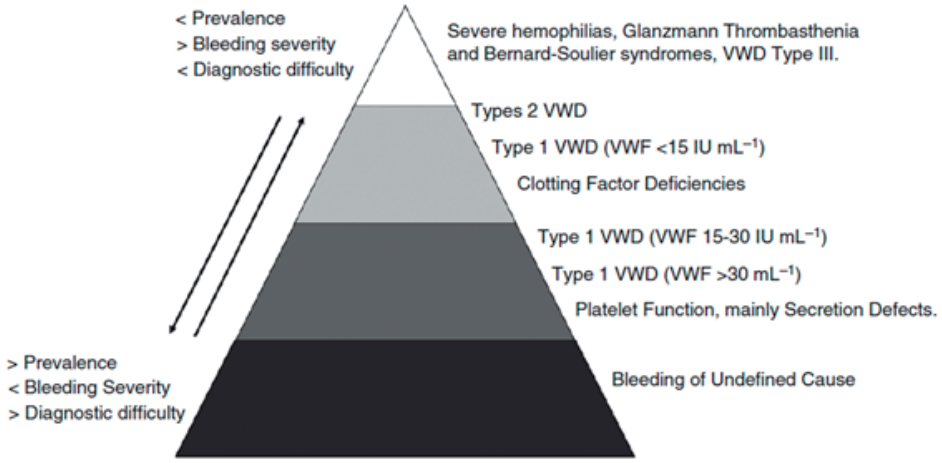
Haemostasis is an interplay between multiple (haemostatic) factors resulting in the formation of a blood clot, preventing blood loss after trauma. Adequate clot formation requires the formation of a platelet plug (primary haemostasis) and clot stabilization and strengthening mediated by fibrin (secondary haemostasis). Furthermore, after wound healing the thrombus needs to be resolved to ensure continuous blood flow (fibrinolysis). Disturbance of this haemostatic balance can result in either hypocoagulability, which is associated with an increased bleeding risk, or hypercoagulability, which is associated with an increased risk of thrombosis.

Diagnosing bleeding disorders

Bleeding disorders can be either inherited or acquired. Inherited bleeding disorders may be due to quantitative of qualitative defects of one or more of the components of the haemostatic system or vessel wall collagen and other matrix components. The most frequently diagnosed bleeding disorders are von Willebrand disease (VWD), with either a reduction of Von Willebrand factor (VWF) – levels or a qualitative defect of VWF, and platelet function disorders (PFDs). Inherited coagulation factor deficiencies including Haemophilia A, caused by a decrease in factor VIII (FVIII), or Haemophilia B, caused by a decrease in factor IX (FIX)) are a much less common cause of a bleeding phenotype.[1,2]

Patients with a bleeding disorder present with various symptoms such as hematomas and easy bruising, mucocutaneous bleeding, and bleeding after trauma or interventions e.g. surgery or tooth extraction.[2] In case of a severe bleeding diathesis, a diagnosis is often easy to make.[3,4] More difficult to diagnose are the mild bleeding disorders. This, because of the only mild bleeding symptoms, which are also seen in otherwise healthy individuals. Moreover, in the general population more than 20% reports at least one bleeding symptom.[5-7] Patients with mild bleeding disorders may not always suffer from bleeding in daily life, but problems may occur after haemostatic challenges, including dental extraction, surgery or delivery. Due to this less severe bleeding phenotype, mild bleeding disorders are often not identified until adulthood.

Furthermore, many clinical studies on patients with (mostly mild) bleeding symptoms have consistently shown that, after wide-ranging laboratory evaluation, in between 47% and 69% of patients no bleeding disorder can be diagnosed.[3] These patients are classified as having Bleeding of Unknown / Undefined Cause (BUC). Patients with BUC seem clinically indistinguishable from those with known MBDs.[1] Currently, it is only possible to speculate on the pathophysiological mechanism of BUC. The accumulation of several subtle impairments in primary and / or secondary haemostatic factors, possibly even without a decrease below normal cut-off levels, could tilt the haemostatic balance toward bleeding.[8]



Adapted from Quiroga T et al 2012[3] and Mezzano D et al 2019[4]

Bleeding assessment tools

The importance of a structured bleeding history is well established in the assessment of a bleeding disorder, and when no laboratory abnormalities are found, the medical history and a standardized bleeding score are important tools for physicians.[9,10] Such tools are known as bleeding assessment tools (BATs) and include a questionnaire to investigate the patients bleeding history. Many studies on bleeding scores in adults have used the MCMDM-1 VWD BS (also known as the Tosetto BS).[11] In 2010, the ISTH Bleeding Assessment Tool (ISTH-BAT) was proposed[12], a validated diagnostic tool that includes data on the frequency and severity of symptoms. The sum of the severity of each reported symptom in patients with bleeding symptoms is known as the individual bleeding score.[13,14] It is known however, that in several studies no differences in bleeding scores have been found between patients with and without an established MBD.[2,15]

Global screening tests in the diagnosis of mild bleeding disorders

Tests that assess the overall haemostatic potential may be of value in screening for and diagnosis of bleeding disorders, as other components of the haemostatic system on blood coagulation are taken into account.[16] This, as most routine haemostatic tests only assess a small part of the total coagulation cascade, with formation of fibrin being the endpoint of these tests.

Rotational thromboelastometry or thromboelastography

Rotational thromboelastometry or thromboelastography provides a graphical representation of blood clot formation and fibrinolysis of whole blood, which therefore includes contributions of erythrocytes, leucocytes and platelets.[17] Viscoelastic changes that occur during

coagulation are measured and the rate of fibrin polymerization and clot strength in whole blood are assessed.[18]

Thrombin generation

The measurement of thrombin generation in plasma has been proposed as a promising approach to globally estimate an individual's coagulation potential and to predict a hypo- or hyper-coagulable state. The area under the thrombin generation curve, or endogenous thrombin potential (ETP), has been used to investigate the risk of both bleeding and thrombosis.[19] This test is not yet used in routine clinical practice.

Plasma clot lysis assay

Increased fibrinolysis of a clot has been associated with bleeding, demonstrated by the bleeding phenotype found in patients with fibrinolysis inhibitor protein deficiencies such as plasminogen activator inhibitor 1 (PAI-1) and alpha-2-antiplasmin (a2-AP).[20, 21,22] Investigation of fibrinolysis is often not part of routine work-up of patients with a bleeding tendency, because deficiencies of fibrinolysis inhibitor proteins are very rare and the pathogenetic role is still doubted. The outcome of the plasma clot lysis assay reflects an overall plasma fibrinolytic potential as it has been shown to be influenced by proteins involved in fibrinolysis and thrombin generation, including plasminogen, a2-AP, PAI-1 and antithrombin.[23,24]

Fibrin clot structure

The fibrin clot structure is the major determinant of the mechanical stability and resistance to lysis of a clot.[25] A fibrin network can have thicker or thinner fibers, large or small pores and increased or decreased fiber density, and these parameters all affect the rate of fibrin dissolution, with clots with thinner fibers being more resistant to fibrinolysis than clots with thicker fibers, as well as being stiffer or more resistant to mechanical deformation.[26] For example, it has been previously shown that coagulation factor deficiencies cause clots with a reduced fiber density and relatively thick fibers[27-32], and that clots made from hemophilic plasmas for example, have altered characteristics making them more susceptible to fibrinolysis.[27,29]

Haemostatic treatment for patients with BUC

Optimal management for prevention and treatment of bleeding in BUC patients is unclear, as only limited evidence on effective and adequate management strategy is available for these patients.[33] As a consequence, these patients may bleed during intervention and may be exposed to unnecessary red blood cell transfusion and blood products following this haemostatic challenge. To date, only one study documented clinical characteristics and response to treatment in BUC, with successful haemostatic outcome in 90% of BUC patients treated with desmopressin (DDAVP) and / or tranexamic acid (TXA).[34] Assuming a multifactorial cause of

bleeding in BUC, with an accumulation of several subtle impairments in primary and / or secondary haemostatic factors, possibly even without a decrease below cut-off levels, could explain, at least in part, why bleeding in these patients may be empirically controlled or attenuated with such diverse therapeutic measures as desmopressin, inhibitors of fibrinolysis (TXA), plasma factors, or platelet transfusions.[3]

Bleeding issues in women with (mild) bleeding disorders and BUC

In several cohort studies, the majority of patients with BUC is female (around 80%)[2], with women having a higher chance of manifest bleeding due to menstrual cycle and childbirth. Therefore, bleeding issues in women include heavy menstrual bleeding (HMB), ovulation bleeding, excessive and prolonged bleeding after miscarriage, and primary and secondary postpartum haemorrhage (PPH). Anatomical rather than haemostatic causes may contribute to these bleeding complications.[5] Other mechanisms, such as the influence of female hormones on skin and muscle possibly leading to easy bruising however, are still largely unknown, but could play a role in both bleeding and thrombotic complications in women.

Diagnosing bleeding disorders in women

Previously reported data consistently show that differences exist in levels of coagulation factors between men and women.[35-40] Currently however, universal routine haemostatic reference ranges are used in most clinical laboratories, with reference ranges being based on healthy male or mixed-population blood donors. Outcome of haemostatic testing based on these universal reference ranges may therefore be incorrect and pose women to an increased bleeding or thrombosis risk throughout life. It is also shown that the diagnosis of a bleeding disorder with only mild haemostatic abnormalities could be influenced, and possible delayed, by the menstrual cycle.[41] With lowest levels of several haemostatic factors found during the menstrual and early follicular phase, these phases seem to be the most optimal timing for haemostatic testing.[41-44]

Postpartum haemorrhage (PPH)

During a normal pregnancy, major physiological changes in haemostasis are seen. Briefly, pregnancy is associated with an increase in concentrations of most coagulation factors, a decrease in concentrations of some of the natural anticoagulants and impaired fibrinolytic activity, which together induce a thrombophilic state, especially in the last trimester.[45,46] These changes in haemostasis, resulting from hormonal and hemodynamic changes, may protect women from fatal haemorrhage during delivery. Postpartum haemorrhage (PPH) is still, however, the major cause of maternal death worldwide, and the prevalence of PPH is steadily increasing in many high-resource countries.[47,48] Primary PPH is traditionally defined as 500 ml blood loss or more within 24 hours after delivery, independent of the mode of delivery.[49,50] PPH can further be classified as minor (500 – 1000 mL) or major (> 1000 mL)

PPH[51], with major PPH being subdivided in moderate (1001 – 2000 mL) and severe (> 2000 mL) PPH.[52,53,54] The most frequent obstetrical causes of PPH are uterine atony, (partially) retained placenta, or perineal trauma (episiotomy and / or lacerations). A history of PPH, advanced maternal age, preeclampsia, macrosomia and multiple gestation are known risk factors for PPH.[55,56] However, the cause may be multifactorial and additional factors may remain unidentified in a significant number of cases. It is known that pre-existing coagulation disorders, such as VWD and carriership of haemophilia, are a risk factor for PPH.[55,57] Even mild haemostatic abnormalities, however, are independently associated with a significantly increased risk for severe PPH, which include decreased levels of fibrinogen, and low von Willebrand factor (VWF) levels.[8] For women with low VWF levels before pregnancy, plasma VWF levels typically increase during normal pregnancy[40] and would be expected to reach the normal range (50-150 IU/dL) for most female patients. However, also low VWF may be associated with PPH despite only mild plasma VWF reductions and normalization of VWF levels during pregnancy.[8,58] The prevalence of inherited bleeding disorders among women presenting with primary PPH is unknown.

Thrombotic disorders, thrombotic complications and treatment

If the coagulation system is not functioning properly, leading to a hypercoagulable state, thrombosis may occur. Thrombosis is the presence of a blood clot in an artery (arterial thrombosis) or vein (venous thrombosis) compromising distal blood flow. Major risk factors for venous thrombosis are immobility and older age[59], but also pregnancy[60,61] and use of oral contraceptives (systemic hormones).[62] Thrombophilia is a tendency to develop thrombosis based on inherited or acquired disorders of blood coagulation or fibrinolysis leading to a prothrombotic state. Inherited deficiency of antithrombin (AT), protein C (PC) and its co-factor, protein S (PS) were the first identified causes of thrombophilia.[63,64] Also, two common genetic polymorphism variants are recognized as additional causes of hypercoagulability: factor V Leiden, causing resistance to the anticoagulant action of activated protein C, and prothrombin G20210A, associated with increased levels of circulating prothrombin.[65,66] Acquired thrombophilia is often caused by the antiphospholipid syndrome, a diagnosis that is defined by a combination of clinical criteria, namely the presence of venous or arterial thrombosis or pregnancy complications, and laboratory criteria, including the presence of lupus anticoagulant (LAC) and / or anticardiolipin (aCL) and / or beta2-glycoprotein (β2GP) antibodies of either the IgG or IgM isotype (or both) on 2 or more occasions at least 12 weeks apart.[67]

Mechanical heart valves and vitamin K antagonists

A major risk factor for arterial thrombosis is the presence of a mechanical heart valve.[68] Mechanical heart valves (MHVs) are more durable than bio-protheses, but also more thrombogenic leading to a substantially high risk of thrombosis and systemic embolism.[68-70]

For this reason long-term management with oral anticoagulant therapy with vitamin K antagonists (VKAs) is recommended in patients with MHVs.[70] Anticoagulant treatment is associated with an increased bleeding risk, especially at supratherapeutic INR levels, and with increased thrombotic risk at subtherapeutic INR levels.[71-74] The quality of VKA treatment is expressed as the percent of time in therapeutic INR range (TTR)[75], which is also correlated to thrombotic and bleeding events during treatment.[76,77] Therefore, stability of INR and high quality of anticoagulation control are essential for safe and effective treatment with VKAs. Predictors of poor anticoagulation control have been studied in the past. Among others, a higher intensity therapeutic range and long intervals between measurements are identified as risk factors for poor anticoagulation control.[78,79] It has also been shown that young age and female sex are predictors of lower quality of anticoagulation control and higher risk of complications during VKA therapy.[80-82] The most important determinant of INR stability during treatment with VKAs is the level of coagulation factor VII (FVII), which is strongly related to the half-life of the used VKA (acenocoumarol versus warfarin).[83,84] Several studies have reported hormone dependent fluctuations of coagulation factor levels during the menstrual cycle, including FVII.[41,85,86]

Aim and outline of the thesis

The overall aim of this thesis is to investigate the diagnostic process and management of patients with bleeding of unknown cause (BUC). More insight in the role of global screening tests in the diagnostic process and in the optimal treatment strategy for these patients may improve long-term clinical outcome. This thesis consists of two parts, with the first part focusing on diagnosis and management of patients with BUC. The second part focusses on female specific health issues in haemostasis and thrombosis, as majority of patients being referred for bleeding symptoms is female (>80%) and women have a higher chance of manifest bleeding due to menstrual cycle and women's ability for child birth.

In **chapter 2**, we will evaluate the management and outcome of haemostatic challenges in patients referred to our tertiary outpatient clinic with a bleeding tendency (n = 462) in retrospect, as only one study documented clinical characteristics and response to treatment in BUC before.[34] In this chapter, the aim is to investigate recommended treatment strategies for different patient categories. In addition, we report on the outcome of surgical procedures and deliveries of patients evaluated at our outpatient clinic for a bleeding tendency, with extensive documentation of bleeding complications, such as major bleeding and major PPH.

It has been suggested that coagulation tests that assess the overall haemostatic potential may be of additive value in screening for and diagnosis of bleeding disorders, as other components of the haemostatic system on blood coagulation are investigated more thoroughly. Therefore, in **chapter 3**, we report a prospective cohort study, in which we included 181 patients referred for analysis of a bleeding tendency to our tertiary outpatient clinic and 76 healthy controls, in order to gain more insight into the pathophysiological mechanisms of

bleeding symptoms in patients with BUC and MBD, and to investigate the diagnostic value of global haemostasis tests in these patients. In this chapter, we investigate the role of these global screening tests (rotational thromboelastometry, thrombin generation, plasma clot lysis assay) in the diagnostic work-up of patients with a bleeding tendency.

Correlations between fibrinogen concentration measured by the Clauss assay and FIBTEM clot firmness parameters (one of the variables found by rotational thromboelastometry) in different patient groups and healthy individuals in a real-life hospital setting are described in **chapter 4**.

In **part II** of this thesis, focusing on **Female specific health issues in haemostasis and thrombosis**, we report on a cohort of women with severe PPH referred to our outpatient clinic for haemostatic evaluation in **chapter 5**. The aim is to explore the value of haemostatic evaluation in patients with severe PPH without a previously diagnosed bleeding disorder, and assessed the prevalence of bleeding disorders in women with severe PPH.

As antifibrinolytic agents, such as TXA, have been proven to be of value during PPH[87], and a mild decreased level of fibrinogen is associated with a significantly increased risk for major PPH[8], a disturbed fibrin clot structure might, at least in part, explain the bleeding risk in women. Fibrin clot structure, however, is not examined routinely in diagnostic testing. In **chapter 6**, we perform a small pilot study where we investigate the fibrin clot structure of women with and without major PPH, by means of Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy.

Because some haemostatic variables are lower in women than in men[35,36], the use of universal reference ranges may be misleading in women. This may have major consequences for future pregnancies and other situations with an increased thrombosis risk throughout life. Therefore, in **chapter 7**, the aim is to investigate the effect of using women-specific reference ranges for thrombophilia-related haemostatic variables. We calculate women-specific reference ranges based on a group of 55 healthy women and compare outcome of thrombophilia investigation between these women-specific reference ranges and routinely used reference ranges, based on healthy male of mixed-population blood donors.

In **chapter 8**, we focus on the other side of the haemostatic balance, namely thrombotic complications, specifically in women with mechanical heart valves using vitamin K antagonists (VKAs). The difference in anticoagulation control between pre- and postmenopausal women, and the impact of cyclic fluctuations of coagulation factors (including FVII) on INR stability and quality of anticoagulation control in women treated with VKAs, has not yet been studied. We aim to assess anticoagulation control in younger women with a MHV on VKA treatment, by comparing quality of anticoagulation control in this group to older women and age-matched men.

Finally, in **chapter 9 and 10**, we will discuss and summarize the findings of this thesis, put them in a clinical perspective and reflect on the implications of these findings for daily patient care as well as future research.

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PART I

The Clinical Relevance and Significance of New Diagnostic Options in patients with an unexplained bleeding tendency – Results from the 'Crescendo-study'

CHAPTER 2

Outcome of surgical interventions and deliveries in patients with bleeding of unknown cause (BUC): an observational study

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ABSTRACT

Introduction Most optimal management for patients with bleeding of unknown cause (BUC) is unknown, as limited data are available.

Objective Evaluate management and outcome of surgical procedures and deliveries in patients with BUC.

Materials and methods All patients ≥ 12 years of age, referred to a tertiary centre for a bleeding tendency, were included. Bleeding phenotype was assessed and haemostatic laboratory work-up was performed. Patients were diagnosed with bleeding of unknown cause (BUC), a mild bleeding disorder (MBD), or an established bleeding disorder (BD). Data on bleeding and treatment during surgical procedures and delivery following diagnosis were collected.

Results Of 380 included patients, 228 (60%) were diagnosed with BUC, 113 (30%) with a MBD, and 39 (10%) with an established BD. In 14/72 (19%) surgical procedures major bleeding occurred and 14/41 (34%) deliveries were complicated by major postpartum haemorrhage (PPH). More specifically, 29/53 (55%) of the BUC patients that underwent surgery received prophylactic treatment to support haemostasis. Despite these precautions, 4/29 (14%) experienced major bleeding. Of BUC patients not treated prophylactically, bleeding occurred in 6/24 (25%). Of pregnant women with BUC, 2/26 (8%) received prophylactic treatment during delivery, one women with and 11 (46%) women without treatment developed major PPH.

Conclusion Bleeding complications are frequent in BUC patients, irrespective of pre- or perioperative haemostatic treatment. We recommend a low threshold approach toward administration of haemostatic treatment in BUC patients, especially during delivery.

INTRODUCTION

Patients with easy bruising, mucosal bleeding, menorrhagia, and disproportionate bleeding after minor injuries, trauma and surgery are frequently referred to a haematologist to diagnose or to rule out an inherited bleeding disorder.[1] Diagnoses in these patients vary and consists of primary haemostasis disorders e.g. von Willebrand disease and platelet disorders, or secondary haemostasis disorders e.g. haemophilia or other rare coagulation factor deficiencies, or disorders of fibrinolysis and collagen disorders. However, in approximately 50% of these referred patients laboratory tests are normal even after extensive testing.[1-4] These patients are diagnosed with bleeding of unknown cause (BUC). The lack of a clear cause of bleeding often leads to uncertainty and insecurity for patients and treating physicians with regard to therapeutic management in case of haemostatic challenges such as dental or surgical procedures, trauma or child birth, as only limited evidence on most effective treatment is available.[5] As a consequence, these patients are regularly under treated or over treated, respectively leading to increased bleeding (risk) or excessive costs.

To our knowledge, only two studies have documented the characteristics and clinical management of BUC patients during therapeutic interventions. Both studies demonstrated that effective prevention or cessation of bleeding occurred in 90% of BUC patients treated with desmopressin and / or tranexamic acid.[5,6] However, we believe that increased insight into this patient group will lead to better tailoring of treatment strategies. Therefore, in a large cohort study, we retrospectively identified patients referred to a tertiary clinic for haemostatic evaluation and diagnosed with either BUC, a mild bleeding disorder (MBD) or an established bleeding disorder (BD). Subsequently, outcomes of surgical procedures and deliveries were evaluated.

METHODS

Study population

All consecutive patients, aged 12 years and older, referred to the outpatient Haematology clinics of the Erasmus University Medical Centre and/or the Erasmus University Medical Centre - Sophia Children's Hospital between 2014 and 2018 for haemostatic screening due to a bleeding tendency or an affected family member with a bleeding disorder, were included in this study. Patients previously diagnosed with a bleeding disorder were excluded. Medical records of all included patients were analyzed and follow-up data regarding surgical procedures and deliveries occurring after referral for haemostatic evaluation were collected. This study was not subject to the Medical Research Involving Human Subjects Act and approved by the Medical Ethics Committee of the Erasmus University Medical Centre Rotterdam.

Bleeding Assessment Tools

The Condensed MCMDM-1 VWD bleeding questionnaire or the ISTH-BAT was used by the (paediatric) haematologist to evaluate bleeding symptoms. The cut-off value for an abnormal score using the Condensed MCMDM-1 VWD bleeding questionnaire is ≥4 for all ages and sex [7]. For the ISTH-BAT, cut-off values for an abnormal score are ≥4 in male adults, ≥6 in female adults and ≥3 in children under 18 years of age [8].

Blood sampling procedure and laboratory assays

Blood sampling was performed using a Vacutainer system (Becton Dickinson) and vials containing either sodium citrate (final concentration 0.109 mol/L) or EDTA (1.8mg/ml, Plymouth). Blood cell count and blood type were determined. Routine coagulation tests aPTT (Actin FS), PT (Thromborel S) and fibrinogen (Thrombin Reagent) were measured on a Sysmex CS5100 (Siemens Healthcare Diagnostics B.V.). Collagen-ADP (C-ADP) and collagen-epinephrine (C-EPI) cartridges were used to measure closure times (CT, seconds) on the PFA-200 (Siemens). Light Transmission Aggregometry (LTA) was performed on a Chrono-Log aggregometer 490 (Stago Benelux B.V.). Von Willebrand factor antigen (VWF:Ag) levels were determined with an in-house ELISA assay, using polyclonal rabbit antihuman VWF antibodies (DakoCytomation) for capturing and detection. Von Willebrand factor collagen binding (VWF:CB) activity was measured by an in-house ELISA assay using bovine Achilles tendon collagen type I for capturing (Sigma-Aldrich) and polyclonal rabbit antihuman VWF antibodies (DakoCytomation) for detecting. Von Willebrand factor activity (VWF:GPIbM) was determined with the INNOVANCE VWF Ac assay (Siemens) on a Sysmex CS5100. FVIII and FIX coagulant activity (FVIII:C/FIX:C) were measured using one-stage clotting assays and derived from (the prolongation of) the clotting time (APTT) measured on the Sysmex CS-5100 (Siemens). FXIII activity was measured using the Berichrom® FXIII kit (Siemens) on the Sysmex CS5100 (Siemens). Alpha 2-antiplasmin was measured using a chromogenic assay (Stachrom, Stago) on the Sysmex CS5100 (Siemens).

Categorization of diagnoses

Based on medical history and laboratory investigation patients were divided into three diagnostic categories:

- Bleeding of unknown cause (BUC): Bleeding was considered of unknown cause based on a clinically relevant bleeding history but no detection of haemostatic abnormalities after extensive laboratory investigation, as described before. [5,9,10]
- Mild BD (MBD): A mild bleeding disorder was considered confirmed if criteria were met for low VWF, a platelet function disorder, or a heterozygous coagulation factor deficiency, further specified as:
    - o *Low VWF* – VWF-activity levels between 0.30-0.50 U/ml and ratio of FVIII:C/VWF:Ag > 0.6 [11];

- *Platelet function disorder not otherwise specified (NOS)*: abnormalities found using light transmission aggregation testing (LTA), not fitting a pattern of any known platelet function disorder [12];
- *Isolated coagulation factor deficiency*: deficiency of a coagulation factor, other than FVIII or FIX, with laboratory criteria as proposed by the European Network of Rare Bleeding Disorders (EN-RBD) [13,14].

• Established BD (BD): A bleeding disorder was confirmed if the identified laboratory abnormalities were in accordance with the definitions of an established bleeding disorder (e.g. von Willebrand disease or haemophilia) stated in national and international guidelines [11,12,15].

Definitions of low, moderate and high-risk surgical procedures and bleeding complications

Definitions of low, moderate and high-risk surgical procedures were defined as described before (supplemental table 1a).[16] Definitions of major bleeding, clinical relevant minor bleeding and (major) postpartum haemorrhage (PPH) are as stated by the ISTH[17,18] and WHO[19] (supplemental table 1b).

Statistics

We used descriptive statistics to summarize baseline characteristic of all patient groups. In case of a skewed distribution, data are presented as median and interquartile range (IQR). Categorical data are presented as numbers with percentages. All analyses were performed with SPSS version 21.0 (IBM, Armonk, NY, USA).

RESULTS

Study group characteristics

Between 2014 and 2018, 481 patients referred for haemostatic evaluation were eligible for inclusion. In total, 101 patients were excluded for various reasons: six patients were lost to follow-up, five patients had a liver disease or Ehlers Danlos, eight patients used medication that interfered with haemostasis and 20 women were pregnant at time of haemostatic evaluation. Sixty-two patients were classified as having no bleeding disorder, based on no bleeding history as judged by the haematologist and normal laboratory results. After exclusion of these patients, 380 patients with a bleeding phenotype remained, of whom 228/380 (60%) were classified as having BUC, 113/380 (30%) had a MBD and 39/380 (10%) had an established BD (figure 1). The median age was 32 years (IQR 20 – 47 years), and 79% was female, with the highest percentage of women found in patients with BUC. Blood type O was present in 38% of patients, and 38% of patients had an abnormal bleeding score at time of haemostatic evaluation, with the highest percentage of both blood type O and abnormal bleeding scores found in patients with a MBD (table 1).

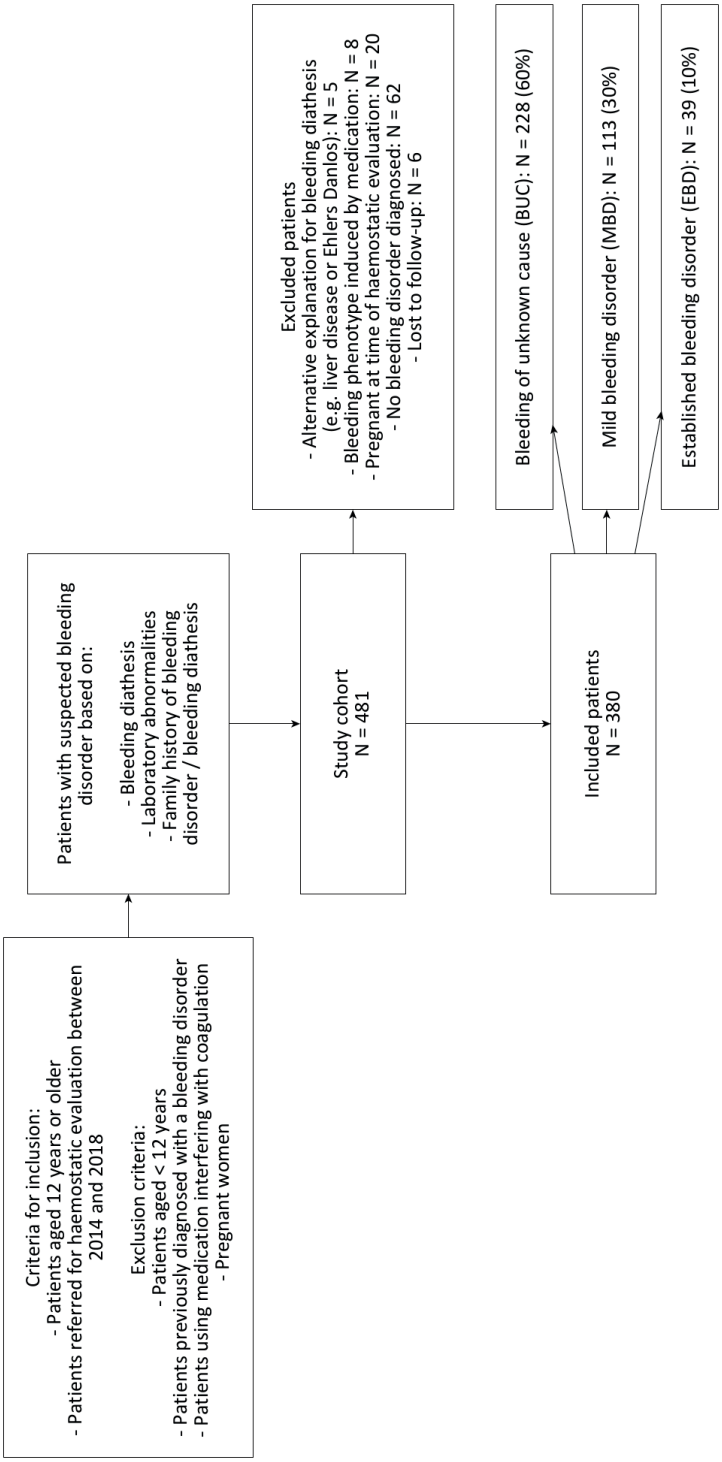

Figure 1. Flowchart of inclusion

Table 1. Study group characteristics

	Total	BUC	MBD	BD
No of patients, n (%)	380 (100%)	228 (60%)	113 (30%)	39 (10%)
Age, median [IQR]	32 [20-47]	33 [23 – 48]	28 [18 – 45]	34 [22 – 53]
Female, n (%)	300 (79%)	186 (82%)	92 (81%)	22 (56%)
Blood group O*, n (%)	146 (44%)	81 (39%)	52 (54%)	13 (45%)
Abnormal bleeding score*, n (%)	145 (45%)	89 (45%)	41 (48%)	15 (42%)
VWF levels, U/ml				
VWF:Ag, median [IQR]	0.90 [0.66 – 1.28]	[0.76 – 1.39]	0.73 [0.53 – 1.14]	0.71 [0.34 – 1.05]
VWF:Act, median [IQR]	0.85 [0.64 – 1.22]	0.96 [0.76 – 1.36]	0.66 [0.48 – 1.02]	0.40 [0.26 – 0.74]
VWF:CB, median [IQR]	0.91 [0.65 – 1.28]	1.05 [0.81 – 1.43]	0.75 [0.49 – 1.07]	0.56 [0.27 – 0.94]
FVIII:C levels, U/ml, median [IQR]	1.16 [0.90 – 1.44]	1.23 [1.08 – 1.62]	1.00 [0.74 – 1.23]	0.62 [0.45 – 1.14]

* Based on available data. Abbreviations: VWF: Von Willebrand Factor, Ag: antigen, Act: activity, CB: collagen-binding, FVIII:C: factor VIII activity.

Surgical procedures complicated by major bleeding

During this study, 72 surgical procedures were performed in 66 patients (Table 2a). In the total study cohort, 19% of surgical procedures was complicated by major bleeding (figure 2).

For BUC patients, 29/53 patients received prophylactic haemostatic treatment. In total, 10/53 (19%) of surgical procedures was complicated by major bleeding. Of the patients receiving treatment, 4/29 (14%) of procedures was complicated by major bleeding, of the patients receiving no treatment, 6/24 (25%) of procedures was complicated by major bleeding. Of the BUC patients receiving treatment, 27 patients received TXA with or without desmopressin. One patient received a platelet transfusion, and one patient received solely clotting FVIII/VWF concentrate. For MBD patients receiving prophylactic treatment (11/15), 2/11 (18%) of procedures was complicated by major bleeding, of the 4/15 patients receiving no treatment, one suffered major bleeding. All four patients with a bleeding disorder (BD) received treatment during surgery (4/4), with one procedure being complicated by major bleeding (figure 3). No statistical significant differences were found between major bleeding in patients with or without haemostatic treatment (figure 3).

Low, moderate and high-risk surgical procedures and major bleeding

Of the 72 surgical procedures, seven procedures were classified as having a high bleeding risk, 36 procedures as having a moderate bleeding risk and 29 procedures as having a low bleeding risk surgical procedures. Four of the seven patients with a high-risk procedures received prophylactic haemostatic therapy, of which one procedure was complicated by major bleeding. Of the patients with moderate risk procedures, 21/36 (58%) received prophylactic haemostatic treatment, of which five procedures (24%) were complicated by major bleeding. Of the patients with low risk procedures, 18/29 (62%) received prophylactic haemostatic treatment. One low risk procedure was complicated by major bleeding (6%)(supplemental figure 1).

Table 2a. Number and characteristics of surgical procedures per patient group

	BUC	MBD	BD
No of surgical procedures	60	21	4
Type of surgical procedure[16]			
High bleeding risk	9	2	2
Moderate bleeding risk	28	9	-
Low bleeding risk	23	10	2
Data complete (treatment and outcome)	53	14	4
Treatment			
None	25	4	-
TXA alone	8	1	-
Desmopressin +/- TXA	18	5	3
Clotting factor concentrate +/- TXA	1	3	1
Platelet transfusion +/- TXA	-	1	-
Desmopressin + platelet transfusion +/- TXA	1	-	-

Abbreviations: BUC: bleeding of unknown cause, MBD: mild bleeding disorder, BD: bleeding disorder, NBD: no bleeding disorder, TXA: tranexamic acid

Table 2b. Number and characteristics of deliveries per patient group

	BUC	MBD	BD
No of deliveries	27	10	6
Vaginal	20	6	6
Caesarian section	7	4	-
Major PPH in medical history	11 (42%)	3 (30%)	2 (33.3%)
Data complete (treatment and outcome)	26	10	5
Treatment			
None	24	7	1
TXA alone	2	-	-
Desmopressin +/- TXA	-	2	-
Clotting factor concentrate +/- TXA	-	-	4
Platelet transfusion +/- TXA	-	1	-
Present peri- and/or postpartum obstetric risk factors for PPH			
Atonic uterus	2	-	1
Retained placenta	5	-	-
Rupture of any kind	12	2	2
Coagulopathy / Preeclampsia	1	2	-
Placental abnormalities	3	1	-

Abbreviations: BUC: bleeding of unknown cause, MBD: mild bleeding disorder, BD: bleeding disorder, NBD: no bleeding disorder, TXA: tranexamic acid, PPH: postpartum hemorrhage

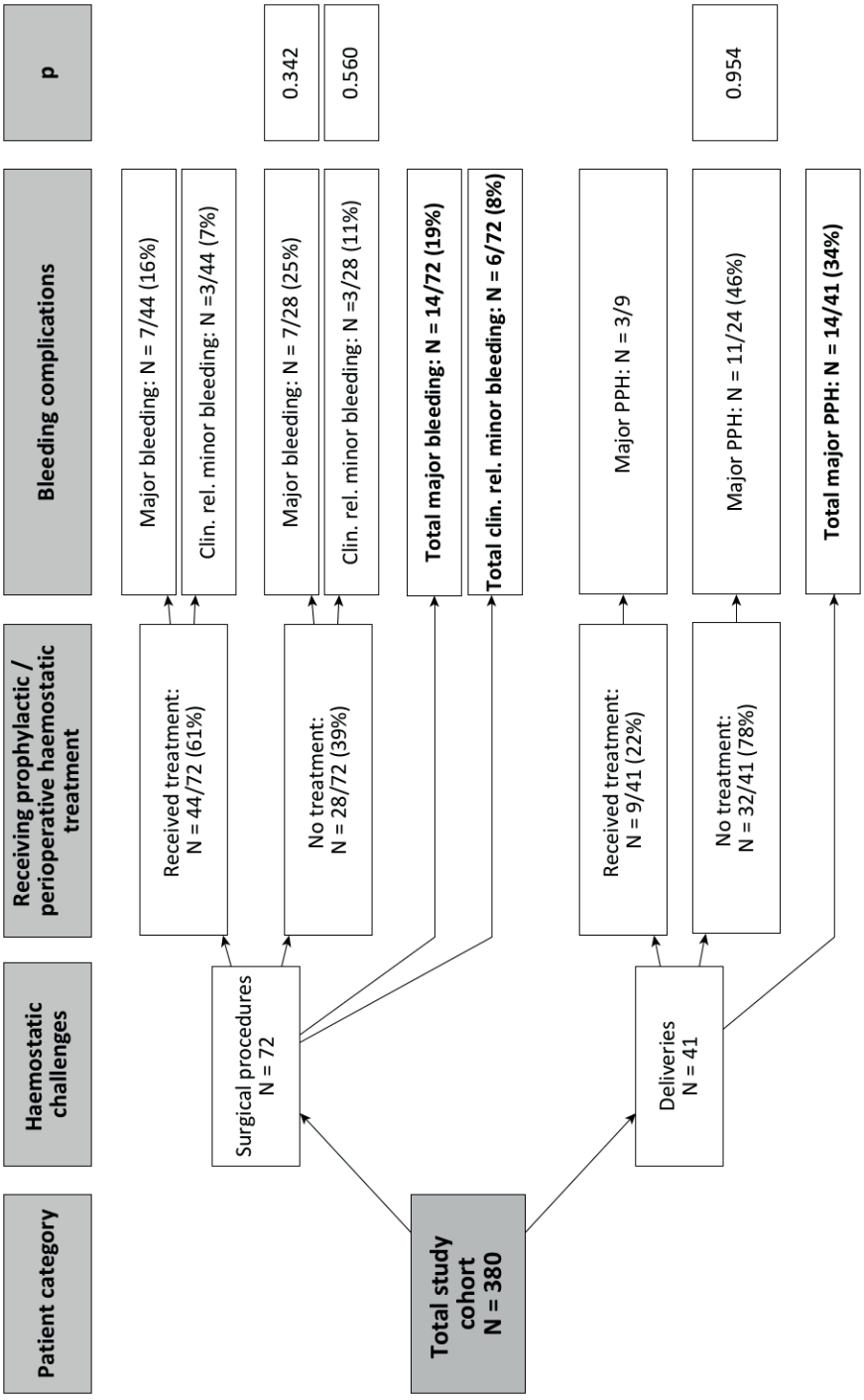

Figure 2. Flowchart of surgical procedures and deliveries (total study cohort)

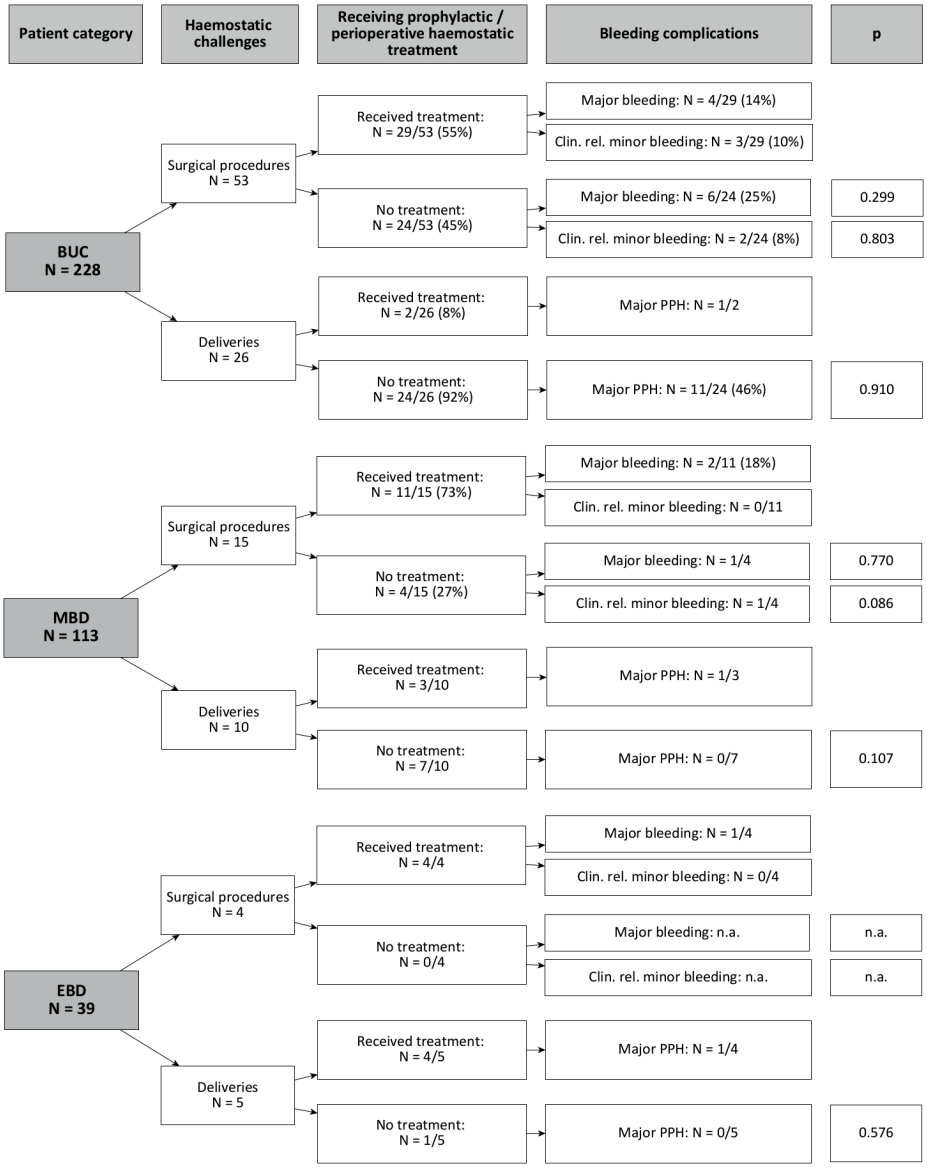

Figure 3. Flowchart of surgical procedures and deliveries (diagnostic subgroups)

Deliveries complicated by major PPH

A total of 43 deliveries in 40 women were registered during the study period, of whom 32/43 (74%) were vaginal deliveries. Based on available data about management and outcome of deliveries following haemostatic evaluation (n=41), 14/41 (34%) of deliveries was complicated by major PPH. In total, 16/43 (37%) of women had major PPH in their medical

history at time of haemostatic evaluation. Of these 16 women, 11/16 (69%) had major PPH at a subsequent delivery during the follow-up period. The patient category with the highest percentage of major PPH was BUC, with 12/26 (46%) of all deliveries being complicated by major PPH. Of these BUC women, only 2/26 (8%) received treatment, being solely tranexamic acid, with 1/2 deliveries being complicated by major PPH. In MBD patients, 3/10 women were treated before delivery, with 1/3 delivery being complicated by major PPH. In BD patients, 4/5 women were treated before delivery, with 1/4 delivery being complicated by major PPH. No statistical significant differences were found between major PPH in women that did received haemostatic treatment and women that did not receive haemostatic treatment during delivery (figure 3). For detailed information about the number of deliveries, treatment per patient category and obstetric risk factors see table 2b.

Bleeding score and major bleeding

Of all the patients with a major bleeding during follow-up (including major PPH) of whom a bleeding score was calculated by the treating physician at time of diagnosis (n=27), 9/27 (33%) had an abnormal bleeding score. Of the patients with a major bleed during surgery of whom a bleeding score was obtained (n=14), 4/14 (29%) had an abnormal bleeding score. Of all the women with major PPH of whom a bleeding score was available (n=13), 5/13 (38%) had an abnormal bleeding score.

Of the patients that had a surgical procedure during follow-up and scored one or higher on the BAT item surgical bleeding (n=38) at time of haemostatic evaluation, indicating previous bleeding during surgery, 8/38 (21%) had a major bleed, versus 6/23 (26%) patients without previous surgical bleeding (BS<1, n=23)($p = 0.650$). Of the women that gave childbirth during follow-up and had a score of 1 or higher on the BAT item PPH (n=15) at time of haemostatic evaluation, indicating PPH in their medical history, 11/15 (73%) had mPPH on follow-up, versus 2/22 (9%) in women without previous PPH (BS<1, n=22)($p<0.01$).

DISCUSSION

This study reports on a cohort of 380 patients referred for analysis of a bleeding tendency to a tertiary outpatient clinic. Sixty percent of these patients were classified as bleeding of unknown cause (BUC). Of the surgical procedures performed in this patient group, 19% was complicated by a major bleed and 46% of the deliveries was complicated by major PPH.

In our study, of the 53 surgical procedures performed in the BUC patient group, 55% of patients received treatment before surgery. In the patients receiving haemostatic therapy, 14% of surgical procedures was complicated by major bleeding and 10% by clinical relevant minor bleeding, indicating that more than 75% of patients experienced no complications during surgery. This is in line with Obaji et al.,[5] and MacDonald et al.,[6] the two studies that

have investigated surgical outcome in a large group of patients with BUC. In these studies haemostatic therapy, consisting of desmopressin and / or tranexamic acid, was administered in almost all BUC patients, with effective haemostatis in 90% of cases. Major bleeding in 19% of patients after surgery in our total study population, as well as in BUC patients, is however much higher than found in the general population. Normally, postsurgical bleeding ranges from 0.6% in orthopaedic procedures[20] to around 3% after tonsillectomy in healthy adults.[21] Furthermore, a large prospective international cohort study of outcomes following elective inpatient surgery in over 44.000 patients, showed that 3% of procedures is complicated by postoperative bleeding, with 0.5% major bleeding.[22] Also, in a study by Mauer et al[23], peri- and postsurgical bleeding was reported in only 6% of included healthy adults. Therefore, we conclude that BUC patients have a higher risk of bleeding compared to the general population.

A striking finding was the occurrence of postpartum haemorrhage in nearly half of the women during childbirth after being analyzed for a bleeding tendency, with one third of deliveries being complicated by major PPH. Of the women with major PPH, the majority had a medical history of major PPH and thus seem to be at higher risk of recurrent PPH. It is known that a history of PPH gives a three-fold higher change of recurrence during subsequent deliveries.[24,25] Also, Stoof et al [26] previously reported that, even in patients with an established BD (VWD or haemophilia carriers) receiving prophylactic haemostatic treatment during delivery, still 34% of women present with PPH.

Of the women with BUC, only 8% received haemostatic treatment pre- or peripartum. A total of 46% of women with BUC however, experienced major PPH after haemostatic evaluation. This percentage is over ten times higher than the incidence of major primary PPH in the general Dutch population (4.5%).[27] In a recently published international, randomised, placebo-controlled trial (WOMAN trial), it was found that tranexamic acid reduces death due to bleeding in women with post-partum haemorrhage without adverse effects.[28] When used as treatment for PPH, it is recommended to give tranexamic acid as soon as possible after bleeding onset. Momentarily, the WOMEN II trial is open aiming to establish if prophylactic tranexamic acid in high-risk women with regard to bleeding is protective.

Because no laboratory abnormalities are identified in patients with BUC, the pathogenesis of the bleeding phenotype remains unknown. Theoretically, bleeding may be caused by a higher fibrinolytic activity, or may be multifactorial, and caused by a number of subtle impairments of primary haemostasis and / or secondary haemostasis, together leading to impaired clot formation. This may explain why bleeding is often controlled effectively by medication that does not compensate for one deficient factor such as desmopressin and tranexamic acid, which have been reported to reduce blood loss and transfusion requirements without thrombotic adverse effects found in several placebo-controlled studies.[28-31]

A recently published consensus report by the EHA[32] states that the aim of a bleeding assessment tool (BAT) is not to demonstrate a strict correlation between any identifiable bleeding

disorder and bleeding score calculated based on a BAT, but to identify those individuals that may benefit from identification as an individual with significant risk of future bleeding. This statement is supported by two major studies that show that a high bleeding score is predictive of postsurgical bleeding for patients with various types of VWD[33] and inherited platelet function disorders.[34] Furthermore Relke et al.,[35] found that a higher BS was associated with a significantly higher risk of future spontaneous bleeding events in BUC patients. Thus, a useful application of BATs could be the ability to identify (BUC) patients who are more likely to bleed excessively during invasive procedures, surgery and childbirth. Unfortunately, we were not able to confirm this viewpoint, as we did not find significant associations between a normal or abnormal bleeding score, or specific items scored on a BAT with surgical outcome and delivery. This may be explained by the small number of procedures and deliveries in our study. Therefore, larger prospective studies must further investigate the value of a BAT in predicting future bleeding complications during haemostatic challenges. We did once again confirm that a history of PPH is a risk factor for future (major)PPH. Based on our own data we recommend that, specifically in women with a history of PPH and in BUC patients, extra awareness for the risk of (recurrent) PPH is needed. A low threshold approach towards bleeding risk during third stage of delivery with early administration of uterotonics and additional haemostatic therapy, such as tranexamic acid, is recommended.

Our study has some limitations. First, our study is a retrospective analysis of real world data on how patients with a bleeding tendency are treated during haemostatic challenges. Prospective trials are needed to confirm our findings and to further investigate the most optimal management strategy for patients with BUC, as they seem to be at higher risk for bleeding complications following surgery and especially delivery. Secondly, a possible selection bias could have occurred due to our status of a tertiary centre, as some surgical procedures and deliveries were performed or managed in other regional hospitals. Information on pre- or perioperative treatment regimens and outcomes were not available for all patients analyzed at our outpatient clinic.

In conclusion, bleeding complications during surgery are frequent in BUC patients, irrespective of pre- or perioperative haemostatic treatment, compared to the general population. In BUC women major PPH occurred frequently during follow-up and a history of PPH was a major risk factor for future (major) PPH. We recommend a low threshold approach toward haemostatic treatment especially during delivery in BUC patients.

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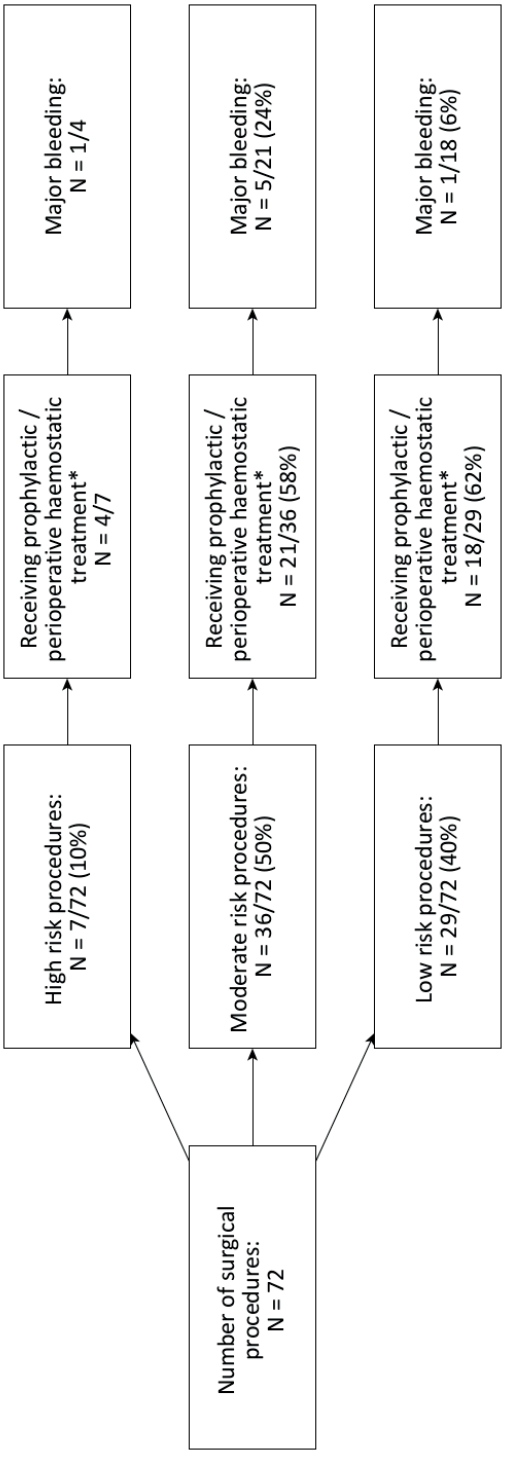

Supplemental figure 1: Number of high, moderate and low risk procedures and % of major bleeding

Supplemental table 1a: Definition and classification of high, moderate, and low bleeding risk surgical procedures

Definition of high, moderate and low bleeding risk surgical procedures, as described before[16]:	
Low bleeding risk surgical procedures	Procedures of the eyes, skin, nose, ears, and distal extremities as well as those pertaining to the dental, perineal, and inguinal areas (eg. inguinal hernia repair, myringotomy, and dilatation and curettage).
Moderate bleeding risk surgical procedures	Procedures of the throat, neck, spine, proximal extremities, genitourinary system, and intra-abdominal areas (eg. tonsillectomy, Cesarean section, splenectomy, cholecystectomy, and hip replacement).
High bleeding risk surgical procedures	Procedures pertaining the intracranial, cardiovascular, and intrathoracic systems (eg. craniotomy and heart valve replacement).

Supplemental table 1b: Definition and classification of (major) bleeding and PPH

Postsurgical and / or postpartum bleedings during follow-up were classified as stated by the ISTH or WHO as follows:	
Major bleeding	Fatal bleeding, and/or symptomatic bleeding in a critical area or organ, and/or bleeding causing a fall in hemoglobin level of 20 g L^{-1} (1.24 mmol L^{-1}) or more, or leading to transfusion of two or more units of whole blood or red cells and/or in case of surgery: surgical site bleeding that required a second intervention or a hemarthrosis of sufficient size as to interfere with rehabilitation by delaying mobilization or delayed wound healing, resulting in prolonged hospitalization or a deep wound infection[18]
Clinically relevant minor bleeding	Any sign or symptom of hemorrhage that does not fit the criteria for the ISTH definition of major bleeding but does meet at least one of the following criteria: 1. Requiring medical intervention by a healthcare professional; 2. Leading to hospitalization or increased level of care; or 3. Prompting a face to face (i.e. not just a telephone or electronic communication) evaluation[17]
Postpartum hemorrhage (PPH)	Estimated blood loss of 500 ml or more within 24 hours after birth (for both surgical as non-surgical childbirth)[19]
Major postpartum hemorrhage (mPPH)	Estimated blood loss of 1000 ml or more within 24 hours after birth[19]

Supplemental table 2. Outcome of BUC patients prophylactically treated with TXA and / or DDAVP

Age	Sex	BS	Diagnosis	Procedure	Risk procedure[16]	Treatment / agent	Outcome
48	F	5 (T)	BUC	Hemithyroidectomy	Medium	Desmopressin	No bleeding
30	F	8 (I)	BUC	Hysterectomy	Medium	Desmopressin	No bleeding
43	F	7 (I)	BUC	Vitrectomy 2x	Low	Desmopressin	No bleeding
40	M		BUC	Polypectomy	Medium	Desmopressin + TXA	No bleeding
40	F	1 (T)	BUC	Closure of nasal septum perforation	Low	Desmopressin + TXA	No bleeding
20	M	3 (T)	BUC	Cruciate ligament reconstruction	Medium	Desmopressin	No bleeding
36	F	5 (I)	BUC	Tooth extraction	Low	Desmopressin	No bleeding
61	F	8 (I)	BUC	Total hip replacement	Medium	Desmopressin + TXA	No bleeding
21	F	6 (I)	BUC	Tooth extraction	Low	Desmopressin + TXA	No bleeding
65	F	13 (I)	BUC	Pelvic organ prolaps procedure	Medium	Desmopressin + TXA	No bleeding
18	F		BUC	Cyst excision from jaw	Low	Desmopressin	No bleeding
61	F	10 (I)	BUC	Hand surgery	Low	Desmopressin	No bleeding
61	F	10 (I)	BUC	Shoulder surgery	Low	Desmopressin	No bleeding
46	F	13 (I)	BUC	Incisional hernia repair	Low	Desmopressin + TXA	No bleeding
61	M	7 (T)	BUC	Total hip replacement	Medium	TXA	No bleeding
47	F	4 (I)	BUC	Adnex extirpation	Medium	TXA	No bleeding
40	M		BUC	Tooth extraction	Low	TXA	No bleeding
63	F	9 (I)	BUC	Sacral nerve stimulator implant	Medium	TXA	No bleeding
72	M	10 (I)	BUC	Tooth extraction	Low	TXA	No bleeding
29	M	5 (I)	BUC	Colono- and gastroscopy + biopsy	Low	TXA	No bleeding
69	F	6 (I)	BUC	Lumpectomy and sentinel node procedure	Medium	TXA	No bleeding
21	F	6 (I)	BUC	Vaginal delivery	N.a.	TXA	Normal bleeding (<500ml)
63	F	11 (I)	BUC	Transurethral resection of the bladder	Medium	Desmopressin	Clinically relevant minor bleeding
59	F	11 (T)	BUC	Polypectomy	Medium	Desmopressin + TXA	Clinically relevant minor bleeding

Supplemental table 2. Outcome of BUC patients prophylactically treated with TXA and / or DDAVP (continued)

Age	Sex	BS	Diagnosis	Procedure	Risk procedure[16]	Treatment / agent	Outcome
46	F	13 (I)	BUC	Resectjon of retroperitoneal sarcoma, adnex extirpation and hysterectomy	High	Desmopressin + TXA	Clinically relevant minor bleeding
30	M	3 (I)	BUC	Extensive osteotomy of the jaw	Low	TXA	Major bleeding
69	F	14 (T)	BUC	Resection of sarcoma, with resection of spleen and pancreas tail	High	Desmopressin + TXA	Major bleeding

Abbreviations: BUC: bleeding of unknown cause, TXA: tranexamic acid, n.a.: not applicable

Supplemental table 3. Outcome of BUC patients receiving no prophylactic treatment

Age	Sex	BS	Diagnosis	Procedure	Risk procedure[16]	Treatment / agent	Outcome
67	F	4 (T)	BUC	Total knee replacement	Medium	No treatment	No bleeding
52	M	0 (T)	BUC	Exicision neurofibroma	Low	No treatment	No bleeding
13	M		BUC	Arthrotomy elbow	Medium	No treatment	No bleeding
29	F	3 (T)	BUC	Endonasal dacryocystorhinostomy	Medium	No treatment	No bleeding
61	F	2 (T)	BUC	Hand surgery / lipoaspiration	Low / low	No treatment	No bleeding
28	F	4 (T)	BUC	Cervical cerclage	Low	No treatment	No bleeding
22	M	2 (T)	BUC	Septoplasty / excision of cyst in maxillary sinus	Low / medium	No treatment	No bleeding
21	F	6 (I)	BUC	Abdominal laporascopy	Low	No treatment	No bleeding
40	M	4 (I)	BUC	Septoplasty and jaw surgery	Low / medium	No treatment	No bleeding
43	F	4 (I)	BUC	Total thyreoidectomy	Medium	No treatment	No bleeding
73	M	1 (I)	BUC	Transsphenoidal hypophysectomy	High	No treatment	No bleeding
36	F	2 (I)	BUC	Surgical removal of cholesteatoma	Low	No treatment	No bleeding
54	F	17 (I)	BUC	Removal of nerve stimulator implant	Medium	No treatment	No bleeding
35	F	4 (I)	BUC	Cystectomy	Medium	No treatment	No bleeding
30	M	3 (I)	BUC	Rhinoplasty	Low	No treatment	No bleeding
15	M		BUC	Evacuation of chronic subdural hematoma	High	No treatment	No bleeding
80	F	2 (T)	BUC	Tooth extraction	Low	No treatment	No bleeding
31	F	8 (I)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
27	F	3 (I)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
28	F	1 (T)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
25	F	5 (I)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
32	F	2 (I)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
32	F	2 (I)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
31	F	5 (I)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)

Supplemental table 3. Outcome of BUC patients receiving no prophylactic treatment (continued)

Age	Sex	BS	Diagnosis	Procedure	Risk procedure[16]	Treatment / agent	Outcome
31	F	6 (T)	BUC	Caesarean section	Medium	No treatment	Normal bleeding (<500ml)
25	F	8 (I)	BUC	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
40	M	3 (I)	BUC	Tooth extraction	Low	No treatment	Clinically relevant minor bleeding
51	F	-1 (T)	BUC	Partial liver resection	High	No treatment	Major bleeding
43	F	3 (I)	BUC	Closure of atrial septal defect	High	No treatment	Major bleeding
63	F	2 (I)	BUC	Ablation of inflammatory breast cancer	Medium	No treatment	Major bleeding
64	F	3 (I)	BUC	Revision total knee replacement	Medium	No treatment	Major bleeding
64	F	3 (I)	BUC	Total knee replacement	Medium	No treatment	Major bleeding
61	F	6 (T)	BUC	Pelvic lymph node dissection, cystectomy, ovariectomy and formation of ileal neobladder	High	No treatment	Major bleeding
30	F	6 (I)	BUC	Vaginal delivery	N.a.	No treatment	PPH (>500ml)
35	F	4 (I)	BUC	Vaginal delivery	N.a.	No treatment	PPH (>500ml)
34	F	4 (T)	BUC	Caesarean section	Medium	No treatment	PPH (>500ml)
32	F	2 (T)	BUC	Vaginal delivery	N.a.	No treatment	Major PPH (>1000ml)
32	F		BUC	Vaginal delivery	N.a.	No treatment	Major PPH (>1000ml)
37	F	3 (T)	BUC	Caesarean section	Medium	No treatment	Major PPH (>1000ml)
28	F	4 (T)	BUC	Caesarean section	Medium	No treatment	Major PPH (>1000ml)
34	F	0 (I)	BUC	Vaginal delivery	N.a.	No treatment	Major PPH (>1000ml)
18	F	4 (I)	BUC	Vaginal delivery	N.a.	No treatment	Major PPH (>1000ml)
30	F	7 (I)	BUC	Caesarean section	Medium	No treatment	Major PPH (>1000ml)
27	F	6 (I)	BUC	Vaginal delivery	N.a.	No treatment	Major PPH (>1000ml)
30	F	4 (I)	BUC	Vaginal delivery	N.a.	No treatment	Major PPH (>1000ml)
26	F	5 (I)	BUC	Vaginal delivery	N.a.	No treatment	Major PPH (>1000ml)
39	F	15 (I)	BUC	Caesarean section	Medium	No treatment	Major PPH (>1000ml)

Abbreviations: BUC: bleeding of unknown cause, n.a.: not applicable

Supplemental table 4a: Outcome of MBD and BD patients prophylactically treated with TXA and / or DDAVP

Age	Sex	BS	Diagnosis	Procedure	Risk procedure[16]	Treatment / agent	Outcome
35	F		MBD	Diagnostic laparoscopy	Low	Desmopressin	No bleeding
22	M	4 (I)	MBD	Sacral nerve stimulator implant	Medium	Desmopressin	No bleeding
23	F	10 (T)	MBD	Tooth extraction	Low	Desmopressin + TXA	No bleeding
17	F		MBD	Cardiac catheterization	High	Desmopressin	No bleeding
56	F	4 (I)	MBD	Bronchoscopy + biopsy	High	Desmopressin + TXA	No bleeding
16	M		MBD	Repair of radial head dislocation	Low	TXA	No bleeding
33	F	6 (I)	MBD	Vaginal delivery	N.a.	Desmopressin	Normal bleeding (<500ml)
55	F	10 (T)	MBD	Bricker urostomy and ileostomy formation	Medium	Desmopressin + TXA	Major bleeding
28	F	5 (T)	MBD	Caesarean section	Medium	Desmopressin + TXA	PPH (>500ml)
46	M	9 (I)	BD	Tooth extraction	Low	Desmopressin + TXA	No bleeding
11	F		BD	Bronchoscopy + biopsy	High	Desmopressin	No bleeding
35	F	2 (I)	BD	Vaginal delivery	N.a.	Desmopressin + TXA	Unknown

Abbreviations: MBD: mild bleeding disorder, BD: bleeding disorder, TXA: tranexamic acid, n.a.: not applicable

Supplemental table 4b: Outcome of MBD and BD patients receiving no prophylactic treatment

Age	Sex	BS	Diagnosis	Procedure	Risk procedure[16]	Treatment / agent	Outcome
53	F		MBD	Several hand surgeries	Low	No treatment	No bleeding
18	M		MBD	Re-orchidopexy	Medium	No treatment	No bleeding
24	F		MBD	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
28	F	4 (I)	MBD	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
26	F	-1 (T)	MBD	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
21	F	7 (I)	MBD	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)
28	F	6 (I)	MBD	Caesarean section	Medium	No treatment	Normal bleeding (<500ml)
44 (479)	M	6 (I)	MBD	Tooth extraction	Low	No treatment	Clinically relevant minor bleeding
67	F	2 (T)	MBD	Total hip replacement	Medium	No treatment	Major bleeding
30	F	5 (I)	MBD	Caesarean section	Medium	No treatment	PPH (>500ml)
37	F	5 (I)	MBD	Caesarean section	Medium	No treatment	PPH (>500ml)
38	F		BD	Vaginal delivery	N.a.	No treatment	Normal bleeding (<500ml)

Abbreviations: MBD: mild bleeding disorder, BD: bleeding disorder, n.a.: not applicable

CHAPTER 3

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

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ABSTRACT

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

Objective Investigate if global haemostasis assays are able to identify patients with a bleeding tendency unexplained by current diagnostic laboratory tests.

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

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

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

INTRODUCTION

Patients with a mild bleeding disorder present with varying symptoms, such as easy bruising, mucocutaneous bleeding, and bleeding after surgery or tooth extraction [1]. However, in the general population bleeding symptoms are reported in more than 20% of healthy individuals [2,3] Therefore, diagnostic evaluation of patients with a bleeding disorder is a challenging process. Application of routine diagnostic laboratory tests in patients with clinically relevant bleeding leaves around 50% of patients without a diagnosis. Subsequently, these patients are classified as patients with bleeding of unknown cause (BUC) [4-6]. Patients with a clinically relevant bleeding phenotype are also regularly diagnosed with mild haemostatic defects, which may not sufficiently explain the patient's bleeding phenotype (mild bleeding disorders, MBD). As a clear diagnosis is lacking in these patients, the most appropriate treatment regimen also remains uncertain [7].

An impaired interplay between several mild haemostatic defects may explain bleeding phenotype in this patient category. Global haemostatic assays may increase insight into the pathogenesis of BUC, as other components of the haemostatic system on blood coagulation are investigated more thoroughly [8]. Rotational thromboelastometry (ROTEM) provides a graphical representation of blood clot formation and fibrinolysis, which includes contributions of erythrocytes, leucocytes and platelets [9]. Measurement of thrombin generation (TG) has also been proposed as a promising approach to globally estimate an individual's coagulation potential and to predict a hypo- or hyper-coagulable state [10]. In addition, investigation of fibrinolysis is often omitted in the routine work-up of patients with a bleeding tendency. It is however known, that clots made from the plasma of hemophilia patient, show altered characteristics and higher susceptibility to fibrinolysis [11,12].

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

MATERIALS AND METHODS

Study population

Patients, aged twelve years or older, referred to the outpatient Haematology and Pediatric Haematology clinics of our tertiary clinics, the Erasmus University Medical Center and Sophia Children's Hospital, for haemostatic screening between June 1st 2016 and March 1st 2018 due to a clinically relevant bleeding tendency were prospectively included. Patients previously diagnosed with a bleeding disorder or diagnosed with an established bleeding disorder after a first laboratory panel (e.g. von Willebrand's disease, haemophilia or platelet disorder),

patients using anticoagulant, antiplatelet or non-steroidal anti-inflammatory drugs, pregnant women, and women less than three months postpartum, were not eligible for study inclusion. A total of 76 sex-matched healthy individuals were included as control group. These healthy individuals were recruited among employees and students of the Erasmus MC University Medical Center. This study was subject to the Medical Research Involving Human Subjects Act and approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2016-218). Written informed consent was obtained from each participant.

Medical bleeding history and Bleeding Assessment Tool

Upon inclusion, all surgical interventions, tooth extractions, obstetric history and detailed family history were documented. A bleeding score (BS), based on the history of bleeding events, was calculated by the ISTH-Bleeding Assessment Tool (ISTH-BAT), with cut-off values ≥ 4 in males, ≥ 6 in females and ≥ 3 in children [13,14].

Blood sampling and laboratory assays

Laboratory tests were performed in a stepwise manner. The first step included a full blood count, ABO blood type, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration according to Von Clauss, determination of VWF antigen (VWF:Ag), activity (VWF:GPIbM) and collagen-binding (VWF:CB), one-stage assay FVIII:C and FIX:C and VWF-multimer analysis in case of low VWF. Platelet function was assessed with the collagen-epinephrine and collagen-ADP cartridges on the platelet function analyzer (PFA-200). As second step, according to the type of abnormalities found, FVII:C, FXI:C and FXIII:C, α2-antiplasmin, and Light Transmission Aggregometry (LTA) were performed. Measurements of VWF:Ag/Act/CB and FVIII:C were repeated at least once.

Blood sampling was performed using the Vacutainer system (Becton Dickinson) containing sodium citrate (final concentration 0.109 mol/L) or EDTA (1.8mg/ml, Plymouth). Citrated blood was centrifuged at 2000g for 10 minutes at room temperature, followed by 14000g for 10 minutes centrifugation of plasma at room temperature. Platelet poor plasma (PPP) samples were stored in aliquots at -80°C until analysis, when indicated. Routine coagulation tests aPTT (Actin FS), PT (Thromborel S) and fibrinogen (Thrombin Reagent) were measured on a Sysmex CS5100 (Siemens Healthcare Diagnostics B.V.). Collagen-ADP (C-ADP) and collagen-epinephrine (C-Epi) cartridges were used to measure closure times (CT, seconds) on the PFA-200 (Siemens). Light Transmission Aggregometry (LTA) was performed on a Chrono-Log aggregometer 490 (Stago Benelux B.V.). VWF:Ag levels and VWF:CB activity were determined with an in-house ELISA assay. VWF activity (VWF:GPIbM) was determined with the INNOVANCE VWF Ac assay (Siemens) on a Sysmex CS5100. FVIII:C and FIX:C was measured using one-stage clotting assays and derived from the prolongation of the clotting time (APTT) measured on the Sysmex CS-5100 (Siemens). FXIII activity was measured using

the Berichrom® FXIII kit (Siemens) on the Sysmex CS5100 (Siemens). Alpha 2-antiplasmin level was measured using a chromogenic assay (Stachrom, Stago) on the Sysmex CS5100 (Siemens).

Rotational thromboelastometry

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

Thrombin Generation

Thrombin generation was assessed using the calibrated automated thrombogram (CAT) assay (Diagnostica Stago, Asnieres, France) in accordance with the manufacturers' instructions, as described previously [15,16]. Briefly, PPP was added to PPP reagent 1 pM TF (PPP Reagent Low, Thrombinoscope B.V., Maastricht, The Netherlands), which consists of a mixture of tissue factor (TF; 1 pM final concentration in plasma) and phospholipids. Plasma of each subject was analyzed in duplicate. Acquisition of thrombin generation parameters was performed using the Thrombinoscope software (Diagnostica Stago, Gennevilliers, France; CAT, Maastricht, The Netherlands). Four parameters were derived from the thrombin generation curve: lag time (min), time to peak (ttpeak, min), endogenous thrombin potential (ETP, nM*min) and peak height (nM).

Plasma clot lysis assay

The plasma clot lysis assay was performed as described before [17,18]. PPP was diluted in buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 1% (w/v) BSA, pH 7.4). The diluted plasma was added to a reaction mixture, containing tissue factor (TF, Innovin, 1000 times diluted; Dade Behring, Marburg, Germany), $CaCl_2$ (17mM), tPA (30ng/ml, Actilyse, Boehringer Ingelheim, Ingelheim am Rhein, Germany), phospholipid vesicles (10µM, Rossix Mölndal, Sweden) and potato carboxypeptidase inhibitor (PCI, an inhibitor of activated TAFI) (30µg/ml) when indicated. The concentrations refer to the final concentrations in the clot. In a microplate reader (Victor™, PerkinElmer, Waltham, MA, USA) the optical density at 405 nm was measured every minute for 300 minutes at 37°C. The clot lysis time (CLT) was the time from midpoint of minimum turbidity to maximum turbidity, which represents clot formation, to the midpoint of maximum turbidity to minimum turbidity, which represents clot lysis. CLTs with and without the addition of PCI were measured in duplicate.

Reference ranges

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

Definition of diagnoses

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

Statistics

We used descriptive statistics to summarize baseline characteristic of the study population. In case of a skewed distribution, data are presented as median and interquartile range (IQR), and compared by a Mann Whitney U test. In case of a normal distribution, data are presented as mean and standard deviation (SD), and compared using an independent sample *t*-test. Categorical data are presented as numbers with percentages and compared using a Pearson Chi-square test. In multiple logistic regression models, we adjusted for age, sex, BMI, platelet count, fibrinogen, VWF, FVIII:C and FXIII:C as appropriate. Outcomes are reported as Odds ratios (ORs) followed by the 95% confidence interval (CI). Multiplicity correction was not performed because of the hypothesis-generating approach of the study. A p-value of < 0.05 was considered statistically significant. All analyses were performed with SPSS version 24.0 (IBM, Armonk, NY, USA).

RESULTS

One hundred and eighty one patients were referred to our hospital with a clinically relevant bleeding tendency and eligible for inclusion, 76 healthy individuals were included as healthy controls. The majority of study participants was female (84% of patients and 86% of healthy controls). Mean age was 33.6 years (SD 17.3) for patients, with 53/181 (29%) adolescent patients ≥ 12 years, and 35.8 years (SD 12.3) for healthy controls, see table 1. For study protocol and flow of inclusion see figure 1.

A total of 120/181 (66%) patients were classified as having BUC. Sixty patients were classified as having a MBD, with platelet function disorders (43%) and low VWF (35%) being

Table 1. Study group characteristics

	Bleeding of Unknown Cause (BUC) (n=121)		Healthy controls (HC) (n=76)	p[§]	Mild bleeding disorder (MBD) (n=60)		p¶
Age, median [IQR]	33 [24 – 50]		32 [26 – 46]	n.s.	20 [15 – 39]		0.001
Adults, n (%)	97 (80%)		76 (100%)	0.000	31 (52%)		0.000
Female, n (%)	105 (87%)		65 (86%)	n.s.	47 (78%)		n.s.
BMI, median [IQR]	26.5 [22.4 – 29.8]		23.6 [21.8 – 27.3]	0.020	24.0 [20.3 – 27.5]		0.047
Bleeding score, median [IQR]	5 [3 – 8]		0 [0 – 2]	0.000	7 [6 – 9]		0.001
Abnormal bleeding score[†], n (%)	65 (54%)		1 (1%)	0.000	51 (88%)		0.000
Blood group O, n (%)	48 (40%)		28 (38%)	0.036	31 (53%)		0.049
Positive family history[‡], n(%)	37 (31%)		0 (0%)	n.a.	28 (48%)		n.s.
Presenting symptom, (%)	Haematomas Postsurgical bleeding Postpartum haemorrhage	31% 18% 15%	n.a.	-	Haematomas Postsurgical bleeding Family history	32% 22% 20%	-
Referring physician, (%)	Haematologist from local hospital General practitioner Gynaecologist	29% 22% 19%	n.a.	-	Paediatrician General practitioner Haematologist from local hospital	35% 24% 22%	-

Data are shown as median and interquartile range [25th – 75th percentile], and number and percentage, as appropriate.

Abbreviations: BMI: body mass index; BUC: bleeding of unknown cause; HC: healthy controls; MBD: mild bleeding disorder; n.a.: not applicable; n.s.: non-significant.

[†]Abnormal bleeding scores: ≥6 for female, ≥4 for male, ≥3 for adolescents.

[‡]1st, 2nd or 3rd degree family member diagnosed with a bleeding disorder or evaluated at a Haematology outpatient department for a bleeding tendency.

[§]Comparison of BUC patients and healthy controls.

[¶]Comparison of BUC patients and patients with a MBD.

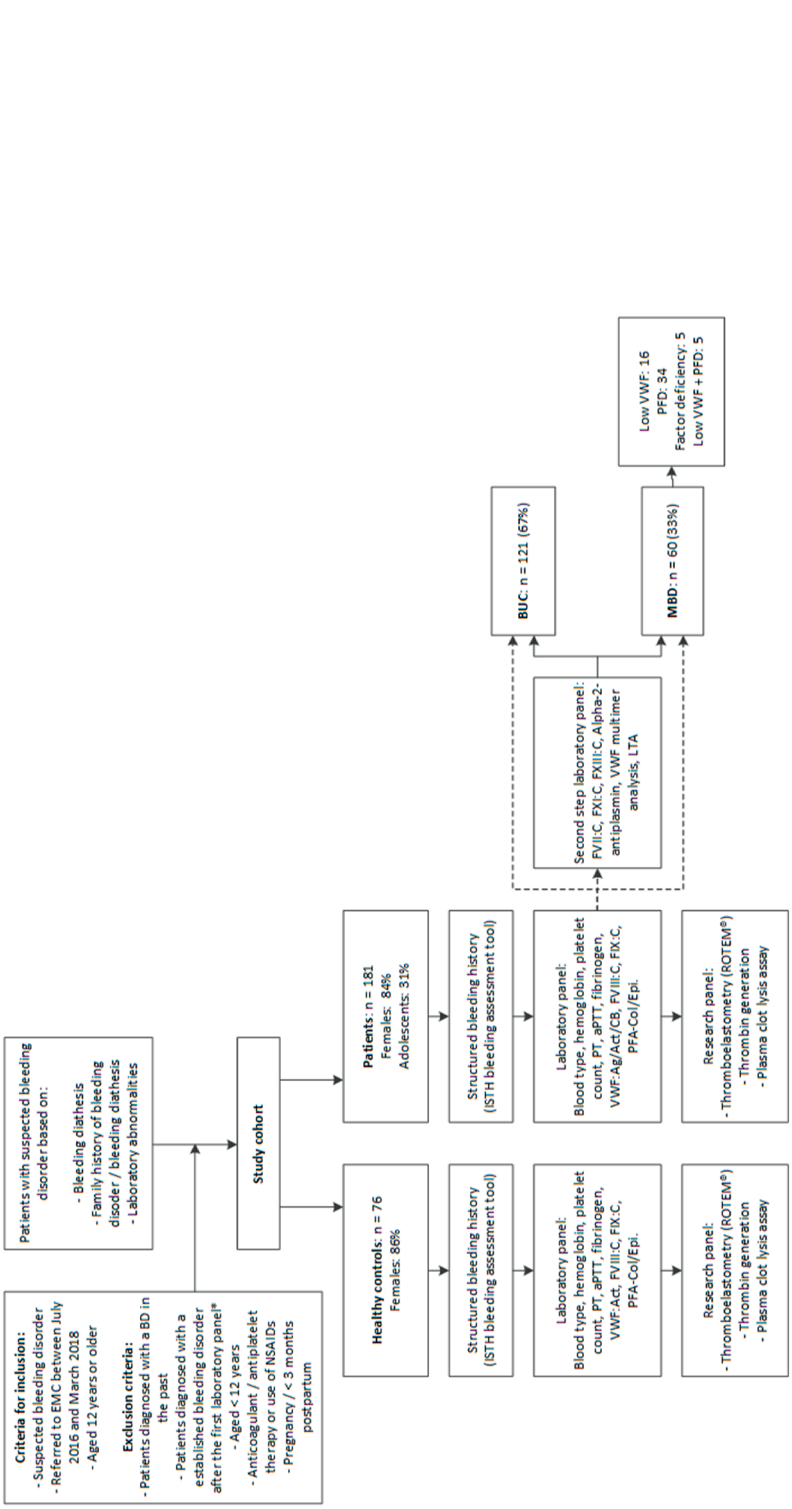

Figure 1. Flowchart of study protocol and inclusion

Abbreviations: PT: prothrombin time; aPTT: activated partial thromboplastin time; VWF: von Willebrand factor; FVIII: factor VIII, FIX: factor IX, PFA-Col/Epi: platelet function analyser collagen / epinephrine; FVII: factor VII; FXI: factor XI; FXIII: factor XIII; LTA: light transmission aggregometry; No BD: no bleeding disorder; BUC: bleeding of unknown cause; MBD: mild bleeding disorder; PFD: platelet function disorder.

most prevalent. BUC patients consisted of a higher percentage of adults (80% versus 52% in MBD, p<0.01), and had a higher median age (33y, IQR 24-50y) than MBD patients (20y, IQR 15-39y, p<0.01). Significantly less BUC patients had blood type O (40%) than MBD patients (53%, p<0.05). BUC patients had a median BS of 5 (IQR 3 – 8), compared to a median BS of 7 (IQR 6 – 9) in patients with MBD (p<0.01), with only 54% of BUC patients presenting with an abnormal BS, compared to 88% in MBD patients (p<0.01) (see table 1).

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

When comparing BUC patients and healthy controls, no statistically significant differences were observed in thromboelastometry variables (see figure 2 and supplemental table 1). When adjusting for age, sex, BMI, platelet count, fibrinogen-, VWF:GPIbM- and FVIII:C-levels by means of logistic regression analysis, no significant differences were found between BUC patients and healthy controls (supplemental table 2). Comparing BUC patients with MBD patients, BUC patients had a significantly decreased clot formation time (CFT) in the EXTEM and INTEM assay, and a significantly increased maximum clot firmness (MCF) in the EXTEM, INTEM and FIBTEM assay (see figure 2 and supplemental table 1).

BUC patients had a significantly longer lagtime (median 7.7 min, IQR: 6.6 – 8.7 min) compared to healthy controls (median 6.9 min, IQR: 6.0 – 8.6 min, p<0.05). Other thrombin generation parameters were not different between BUC patients and healthy controls (supplemental table 1). When adjusting for age, sex, BMI, platelet count, fibrinogen- and FXIII:C-levels by means of logistic regression analysis, also no significant differences were found between BUC patients and healthy controls (supplemental table 2). In MBD patients, impaired thrombin generation was found, with a significantly decreased ETP and peak height compared to BUC patients (see figure 2) and healthy controls.

Remarkably, a significant longer CLT in BUC patients (PCI- median 60.3 min, IQR 54.7 - 66.0 min and PCI+ 41.3 min, IQR 38.0 - 46.2min) was found compared to healthy controls (PCI- median 57.4 min, IQR 53.9 - 61.7min and PCI+ 38.9, IQR 36.3 - 42.5min, p=0.03 and p<0.01 respectively)(supplemental table 1). However, when adjusting for age, sex, BMI, platelet count, fibrinogen- and FXIII:C-levels by means of logistic regression analysis, no significant differences were found between BUC patients and healthy controls (supplemental table 2). Overall, no differences were found in CLT between BUC patients and MBD patients, both with and without adjustment for age, sex, BMI, platelet count, fibrinogen- and FXIII:C-levels.

In both thromboelastometry, as well as thrombin generation and clot lysis time variables, no additional significant differences were found between BUC and MBD patients, after excluding patients with a normal bleeding score (see table 2 and supplemental table 1).

Table 2. Haemostatic variables in different patient groups and healthy controls

	n[†]	BUC	n[†]	HC	p[§]	n[†]	MBD	p[¶]
Hemoglobin, mmol/L	115	8.1 [7.8 – 8.8]	75	8.2 [7.8 – 8.7]	n.s.	55	8.6 [8.1 – 9.0]	0.015
	61[‡]	8.1 [7.9 – 8.8]	n.a.		n.s.	47[‡]	8.7 [8.2 – 9.1]	0.006
Platelet count, 10^9/L	113	272 [236 – 317]	75	253 [224 – 300]	n.s.	58	248 [208 – 283]	0.010
	59[‡]	276 [229 – 328]	n.a.		n.s.	49[‡]	238 [207 – 278]	0.012
PT, sec	116	11.9 [11.4 – 12.5]	76	11.9 [11.5 – 12.6]	n.s.	56	11.7 [11.4 – 12.7]	n.s.
	61[‡]	11.7 [11.2 – 12.5]	n.a.		n.s.	48[‡]	11.7 [11.4 – 12.6]	n.s.
APTT, sec	121	25 [23 – 26]	76	25 [23 – 26]	n.s.	60	26 [24 – 27]	0.010
	63[‡]	25 [24 – 26]	n.a.		n.s.	51[‡]	26 [24 – 27]	0.026
PFA, sec	117	137 [118 – 160]	76	136 [109 – 157]	n.s.	52	151 [126 – 176]	n.s.
	63[‡]	137 [118 – 159]	n.a.		n.s.	44[‡]	151 [123 – 173]	n.s.
Fibrinogen, g/L	120	2.8 [2.4 – 3.4]	76	2.7 [2.3 – 3.4]	n.s.	58	2.7 [2.4 – 3.2]	n.s.
	64[‡]	2.8 [2.4 – 3.3]	n.a.		n.s.	49[‡]	2.7 [2.4 – 3.3]	n.s.
VWF:Ag, U/ml	120	0.98 [7.3 – 1.22]	n.a.	n.a.	n.a.	59	0.73 [0.55 – 1.04]	0.000
	64[‡]	0.91 [0.73 – 1.18]	n.a.		n.a.	50[‡]	0.79 [0.79 – 1.02]	0.003
VWF:Act, U/ml	121	0.89 [0.75 – 1.22]	76	0.93 [0.77 – 1.24]	n.s.	59	0.74 [0.54 – 1.02]	0.001
	65[‡]	0.89 [0.76 – 1.12]	n.a.		n.s.	50[‡]	0.79 [0.54 – 1.04]	0.026
VWF:CB, U/ml	120	0.87 [0.70 – 1.09]	n.a.	n.a.	n.a.	59	0.69 [0.48 – 0.96]	0.000
	64[‡]	0.84 [0.70 – 1.07]	n.a.		n.a.	50[‡]	0.70 [0.52 – 0.97]	0.002
FVIII:C, U/ml	121	1.20 [1.05 – 1.40]	75	1.23 [1.13 – 1.53]	n.s.	59	1.06 [0.79 – 1.21]	0.000
	65[‡]	1.18 [1.07 – 1.34]	n.a.		n.s.	50[‡]	1.08 [0.84 – 1.22]	0.001
FVII:C, U/ml	46	0.88 [0.78 – 1.10]	40	0.98 [0.80 – 1.16]	n.s.	26	0.91 [0.74 – 1.13]	n.s.
	26[‡]	0.86 [0.69 – 1.16]	n.a.		n.s.	22[‡]	0.91 [0.73 – 1.13]	n.s.
FIX:C, U/ml	109	1.05 [0.96 – 1.15]	40	1.02 [0.94 – 1.17]	n.s.	58	1.01 [0.92 – 1.13]	n.s.
	62[‡]	1.08 [0.97 – 1.18]	n.a.		n.s.	49[‡]	1.00 [0.90 – 1.15]	0.040

FXI:C, U/ml	107	1.05 [0.96 – 1.15]	40	1.07 [0.98 – 1.16]	n.s.	57	1.00 [0.93 – 1.12]	n.s.
	59‡	1.06 [0.99 – 1.17]	n.a.		n.s.	48‡	1.00 [0.93 – 1.12]	n.s.
FXIII:C, U/ml	112	1.28 [1.11 – 1.41]	40	1.30 [1.08 – 1.38]	n.s.	59	1.23 [1.06 – 1.31]	n.s.
	62‡	1.23 [1.04 – 1.42]	n.a.		n.s.	50‡	1.23 [1.06 – 1.33]	n.s.
Alpha-2-antiplasmin, U/ml	88	1.15 [1.05 – 1.21]	n.a.	n.a.	n.a.	42	1.15 [1.04 – 1.19]	n.s.
	53‡	1.15 [1.09 – 1.22]	n.a.		n.a.	37‡	1.14 [1.03 – 1.19]	n.s.

Data are shown as median and interquartile range [25th – 75th percentile].

Abbreviations: BUC: bleeding of unknown cause; HC: healthy controls; MBD: mild bleeding disorder; PT: prothrombin time; APTT: activated partial thromboplastin time; PFA: platelet function analyzer; VWF:Ag: von Willebrand factor antigen; VWF:Act: von Willebrand factor activity; VWF:CB: von Willebrand factor collagen binding; FVIII:C: factor VIII activity; FVII:C factor VII activity; FIX:C: factor IX activity; FXI:C: factor XI activity; FXIII:C; factor XIII activity; n.a.: not applicable; n.s.: non-significant.

† Based on available data;

‡ No of patients with abnormal bleeding score;

§Comparison of BUC patients and healthy controls;

¶Comparison of BUC patients and patients with a MBD.

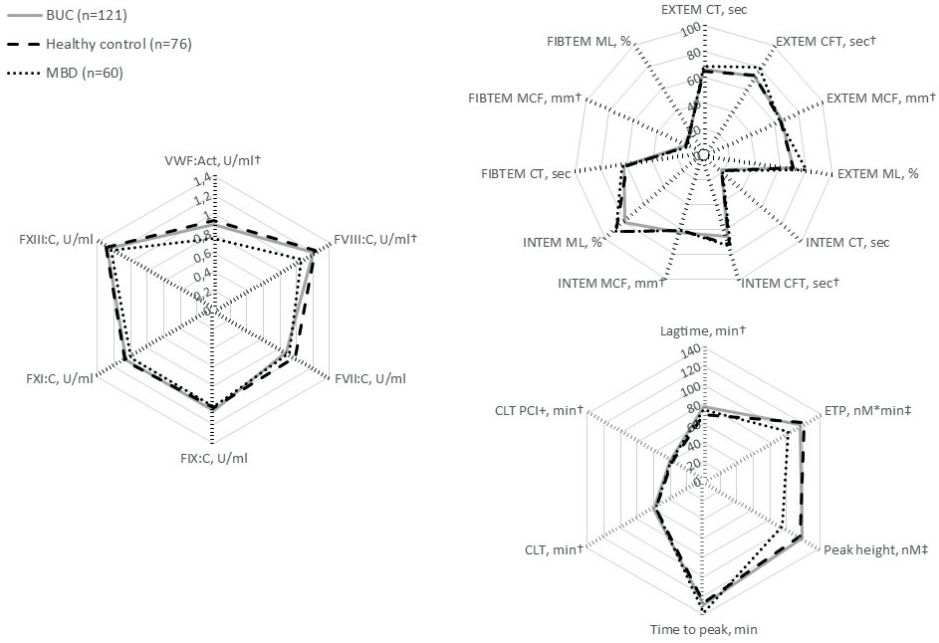

Figure 2. Haemostatic, thromboelastometry, thrombin generation and plasma clot lysis assay variables in patients and healthy controls

EXTEM ML x 10; INTEM CT / 10; INTEM ML x 10; FIBTEM ML x 10; Lagtime x 10; ETP / 10; Time to peak x 10.

Abbreviations: BUC: bleeding of unknown cause; MBD: mild bleeding disorder; VWF: Von Willebrand factor; FVIII:C: factor VIII activity; FVII:C factor VII activity; FIX:C: factor IX activity; FXI:C: factor XI activity; FXIII:C: factor XIII activity; CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness; ML: maximum lysis; ETP: endogenous thrombin potential; CLT: clot lysis time; PCI: potato carboxypeptidase inhibitor. †p<0.05, BUC patients compared to healthy controls. ‡p<0.05, BUC patients compared to MBD patients.

The ETP was significantly lower in patients with an abnormal bleeding score (median 1223nM*min, IQR: 923 - 1516nM*min) compared to patients with a normal bleeding score (median 1055nM*min, IQR: 828 - 1363nM*min, p=0.046). Furthermore, patients with an abnormal bleeding score had a significantly longer CT (median 65min, IQR: 58 - 72min) in the EXTEM-assay and significantly lower MCF (median 16mm, IQR: 13 - 19mm) in the FIBTEM-assay compared to patients with a normal score (CT-EXTEM: median 67min, IQR: 63 - 73min, p=0.048 and MCF-FIBTEM: median 15mm, IQR: 11 - 18mm, p=0.04). Plasma clot lysis time was comparable in patients with an abnormal and normal bleeding score (see figure 3 and supplemental table 4).

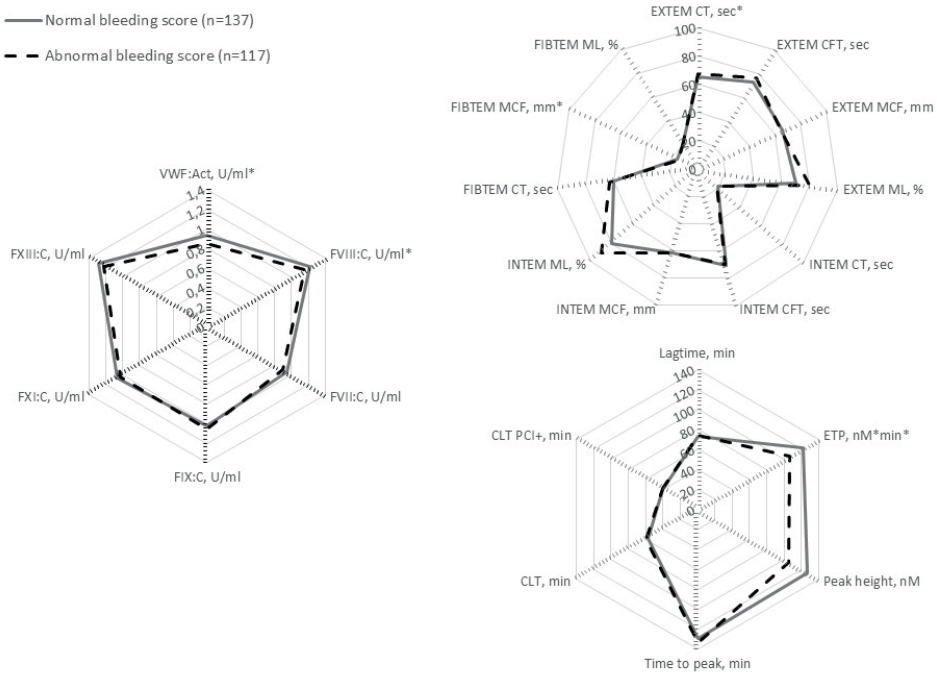

Figure 3. Haemostatic, thromboelastometry, thrombin generation and plasma clot lysis assay variables based on bleeding score
EXTEM ML x 10; INTEM CT / 10; INTEM ML x 10; FIBTEM ML x 10; Lagtime x 10; ETP / 10; Time to peak x 10.
Abbreviations: VWF: Von Willebrand factor; FVIII:C: factor VIII activity; FVII:C factor VII activity; FIX:C: factor IX activity; FXI:C: factor XI activity; FXIII:C: factor XIII activity; CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness; ML: maximum lysis; ETP: endogenous thrombin potential; CLT: clot lysis time; PCI: potato carboxypeptidase inhibitor. *p<0.05.

DISCUSSION

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

We found that rotational thromboelastometry variables are within reference ranges in BUC patients and do not differ from healthy controls and MBD patients. Our results are in line with those recently described by Wieland Greguare-Sander et al. [25], and support their conclusion that there is no support for the additive value of rotational thromboelastometry for screening and diagnosing patients with a (mild) bleeding tendency. Thrombin generation has been applied regularly to investigate bleeding risk in patients with a bleeding disorder [26,27]. In this study, besides a significant longer lag time in BUC patients, thrombin generation

parameters did not differ between patients with BUC and healthy controls, as also shown in previous studies [28,29]. This finding was however, in contrast with recently published data [24], in which all the TG variables in BUC patients were found to be significantly different from healthy controls. Patients with MBDs did show a significantly impaired thrombin generation, with a decreased endogenous thrombin potential (ETP) and peak height. This finding is remarkably however, as the used thrombin generation is a reflection of secondary haemostasis, and most patients in the MBD group are diagnosed with a disorder of primary haemostasis. In addition, no evidence was found supporting a systemic hyperfibrinolytic capacity in BUC patients. In contrast, we found that clot lysis time was significantly prolonged in BUC patients compared to healthy controls, in line with previously published data [30,31], hereby carefully rejecting hyperfibrinolysis as underlying pathophysiological mechanism for BUC.

Several studies have shown that between 47 and 69% of patients will remain undiagnosed after extensive and repeated laboratory testing [1,4]. When no laboratory abnormalities are found, the medical history and a bleeding score are important tools for physicians [6,32]. However, ISTH-BAT has shown to only have a limited role, as a normal score was present in 44% of BUC patients and in 21% of MBD patients. Therefore, a BAT should only serve as one of the many diagnostic tools available in the diagnostic work up of these patients.

We confirm one of the main findings by Gebhart et al., namely, that the majority of patients being referred for bleeding symptoms is female (> 80%) and that more women than men are categorized with BUC, hereby possible affirming that there is a sex-related difference in BUC-rate [1]. Women have a higher chance of manifest bleeding due to menstrual cycle and women's ability for childbirth. Other mechanisms however, such as the influence of female hormones on skin and muscle possibly leading to easy bruising, are still largely unknown [33]. We also showed that patients with BUC were significantly older than patients with a diagnosed MBD. It has been shown that several haemostatic factors increase with age [34]. This may explain that no abnormalities were found in this 'older' subgroup at time of analysis. In addition, the role of comorbidities can be more pronounced in an older population, for example the influence of age and comorbidities on skin and vessels, possibly causing easy bruising or perioperative bleeding [35]. To our knowledge, this is also one of the first studies to report BUC in adolescents, with a higher percentage of adolescents being diagnosed with a MBD than adults.

Our study has some limitations. First, one cannot exclude a possible referral bias for adolescents, with investigations possible being delayed or abandoned if the bleeding score was not very high. This might explain the increased rate of adolescents as well as the higher bleeding scores in the MBD patient group. We performed LTA for investigation of platelet function disorders. An influence of medication on platelet function cannot be ruled out completely. For example, we did not exclude patients using selective serotonin re-uptake inhibitors (SSRI's), which are shown to reduce platelet function [36,37]. Due to the circadian rhythm of plasminogen activator inhibitor-1 (PAI-1), which inhibits fibrinolysis and increases

in the morning [38], we attempted to collect blood for plasma clot lysis assay in the afternoon. Unfortunately, this was not always possible due to logistic reasons.

Additional studies on patients without a clear diagnosis are required. In the near future, advanced techniques such as Next Generation Sequencing (NGS)-based gene panels [39] or Whole Exome Sequencing (WES) [40] may lead to discoveries of novel haemostatic modifiers. However, translating these results will provide a next challenge due to multi-interpretable and uncomprehensive findings such as variants of unknown significance (VUS).

CONCLUSION

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

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Supplemental table 1. Rotational thromboelastometry, thrombin generation and plasma clot lysis variables in different patient groups and healthy controls

		n[†]	BUC	n[†]	HC	p[§]	n[†]	MBD	p[¶]
ROTEM	EXTEM – CT, min	89	66 [62 – 71]	75	64 [58 – 73]	n.s.	48	68 [62 – 74]	n.s.
		53[‡]	67 [63 – 72]			n.s.	43[‡]	67 [62 – 74]	n.s.
	EXTEM – CFT, min	89	72 [60 – 90]	75	73 [64 – 88]	n.s.	48	80 [75 – 95]	0.002
		53[‡]	70 [60 – 92]			n.s.	43[‡]	80 [75 – 95]	0.006
	EXTEM – MCF, mm	89	66 [62 – 69]	75	65 [62 – 69]	n.s.	48	64 [61 – 66]	0.002
		53[‡]	66 [62 – 71]			n.s.	43[‡]	63 [61 – 66]	0.007
	EXTEM – ML, %	89	7 [5 – 9]	75	7 [5 – 10]	n.s.	48	8 [6 – 10]	n.s.
		53[‡]	7 [5 – 9]			n.s.	43[‡]	8 [6 – 10]	n.s.
	INTEM – CT, min	89	184 [167 – 198]	75	187 [170 – 204]	n.s.	50	189 [171 – 202]	n.s.
		53[‡]	184 [169 – 198]			n.s.	45[‡]	184 [169 – 201]	n.s.
	INTEM – CFT, min	89	66 [60 – 79]	75	72 [62 – 82]	n.s.	50	74 [67 – 86]	0.008
		53[‡]	65 [58 – 85]			n.s.	45[‡]	75 [68 – 78]	0.021
	INTEM – MCF, mm	89	63 [59 – 66]	75	61 [59 – 65]	n.s.	50	61 [57 – 63]	0.024
		53[‡]	63 [59 – 66]			n.s.	45[‡]	61 [57 – 63]	n.s.
	INTEM – ML, %	89	8 [6 – 10]	75	9 [7 – 12]	n.s.	50	9 [7 – 11]	n.s.
		53[‡]	9 [6 – 10]			n.s.	45[‡]	10 [7 – 11]	n.s.
	FIBTEM – CT, min	88	62 [57 – 68]	75	60 [55 – 70]	n.s.	49	64 [57 – 74]	n.s.
		52[‡]	63 [57 – 70]			n.s.	44[‡]	65 [57 – 74]	n.s.
	FIBTEM – MCF, min	88	16 [13 – 19]	75	15 [12 – 19]	n.s.	49	14 [11 – 16]	0.003
		52[‡]	16 [12 – 18]			n.s.	44[‡]	14 [11 – 16]	0.036
	FIBTEM – ML, %	88	2 [0 – 5]	75	2 [0 – 6]	n.s.	49	2 [0 – 6]	n.s.
		52[‡]	2 [0 – 5]			n.s.	44[‡]	2 [0 – 6]	n.s.
TG	Lagtime, min	119	7.7 [6.6 – 8.7]	74	6.9 [6.0 – 8.6]	0.046	53	7.4 [6.7 – 8.4]	n.s.
		64[‡]	7.7 [6.3 – 8.7]			n.s.	45[‡]	7.4 [6.7 – 8.5]	n.s.

Supplemental table 1. Rotational thromboelastometry, thrombin generation and plasma clot lysis variables in different patient groups and healthy controls (continued)

		n†	BUC	n†	HC	p§	n†	MBD	p¶
	ETP, nM* min	119	1164 [912 – 1478]	74	1208 [862 – 1494]	n.s.	53	1024 [767 – 1331]	0.022
		64‡	1126 [869 – 1420]			n.s.	45‡	1013 [766 – 1313]	n.s.
	Peak height, nM	119	119 [93 – 162]	74	116 [89 – 170]	n.s.	53	95 [70 – 137]	0.004
		64‡	111 [91 – 152]			n.s.	45‡	95 [70 – 135]	0.039
	Time to peak, min	119	13.0 [11.9 – 14.3]	74	12.7 [11.3 – 13.8]	n.s.	53	13.8 [12.2 – 14.7]	n.s.
		64‡	13.3 [11.9 – 14.3]			n.s.	45‡	13.4 [12.1 – 14.7]	n.s.
CLT	Plasma clot lysis time, min	117	60.3 [54.7 – 66.0]	74	57.4 [53.9 – 61.7]	0.039	52	57.8 [53.4 – 62.6]	n.s.
		62‡	61.3 [54.9 – 67.8]			n.s.	44‡	57.8 [53.5 – 62.0]	n.s.
	Plasma clot lysis time^{PCI+}, min	117	41.3 [38.0 – 46.2]	74	38.9 [36.3 – 42.5]	0.004	52	40.1 [35.7 – 44.5]	n.s.
		62‡	41.4 [37.8 – 46.9]			0.026	44‡	40.1 [35.7 – 44.3]	n.s.
	Ratio CLT / CLT^{PCI+}	117	0.69 [0.67 – 0.72]	74	0.68 [0.66 – 0.71]	n.s.	52	0.69 [0.66 – 0.71]	n.s.
		62‡	0.69 [0.66 – 0.71]			n.s.	44‡	0.69 [0.67 – 0.71]	n.s.

Data are shown as median and interquartile range [25th – 75th percentile].

Abbreviations: BUC: bleeding of unknown cause; HC: healthy controls; MBD: mild bleeding disorder; TG: thrombin generation; CLT: clot lysis time; CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness; ML: maximum lysis; ETP: endogenous thrombin potential; CLT: clot lysis time; PCI: potato carboxypeptidase inhibitor; n.s.: non-significant.

† Based on available data;

‡ No of patients with abnormal bleeding score;

§Comparison of BUC patients and healthy controls;

¶Comparison of BUC patients and patients with a MBD.

Supplemental table 2. Differences between BUC patients and healthy controls based on logistic regression modelling

Outcome	Predictor†		Odds Ratio	95% CI	p
BUC	EXTEM	CT (min)	0.993	0.968 – 1.019	n.s.
		CFT (min)	1.007	0.985 – 1.029	n.s.
		MCF (mm)	0.927	0.828 – 1.039	n.s.
		ML (%)	0.984	0.949 – 1.021	n.s.
	INTEM	CT (min)	0.995	0.983 – 1.008	n.s.
		CFT (min)	0.998	0.983 – 1.014	n.s.
		MCF (mm)	1.001	0.956 – 1.049	n.s.
		ML (%)	0.987	0.960 – 1.013	n.s.
	FIBTEM	CT (min)	0.990	0.966 – 1.015	n.s.
		MCF (mm)	1.024	0.912 – 1.151	n.s.
		ML (%)	1.027	0.939 – 1.124	n.s.

†Corrected for sex, age, BMI, plateletcount, fibrinogen- and FVIII:C-levels.

Outcome	Predictor†		Odds Ratio	95% CI	p
BUC	Thrombin generation	Lagtime (min)	1.250	0.920 – 1.697	n.s.
		ETP (nM*min)	1.000	0.999 – 1.001	n.s.
		Peakheight (nM)	0.998	0.993 – 1.003	n.s.
		Time to peak (min)	1.153	0.937 – 1.418	n.s.

†Corrected for sex, age, BMI, fibrinogen- and FXIII:C-levels.

Outcome	Predictor†		Odds Ratio	95% CI	p
BUC	Plasma clot lysis	CLT (min)	0.994	0.963 – 1.025	n.s.
		CLT PCI+ (min)	0.989	0.938 – 1.043	n.s.

†Corrected for sex, age, BMI, fibrinogen- and FXIII:C-levels.
Abbreviations: CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness; ML: maximum lysis; ETP: endogenous thrombin potential; CLT: clot lysis time; PCI: potato carboxypeptidase inhibitor; n.s.: non-significant.

Supplemental table 3. Differences between BUC patients and MBD patients based on logistic regression modelling

Outcome	Predictor†		Odds Ratio	95% CI	p
BUC	EXTEM	CT (min)	1.007	0.939 – 1.080	n.s.
		CFT (min)	1.026	0.992 – 1.026	n.s.
		MCF (mm)	0.887	0.748 – 1.050	n.s.
		ML (%)	1.008	0.957 – 1.061	n.s.
	INTEM	CT (min)	1.002	0.982 – 1.023	n.s.
		CFT (min)	1.002	0.972 – 1.032	n.s.
		MCF (mm)	1.013	0.927 – 1.108	n.s.
		ML (%)	0.993	0.948 – 1.040	n.s.
	FIBTEM	CT (min)	0.964	0.908 – 1.023	n.s.
		MCF (mm)	0.783	0.619 – 0.991	0.042
		ML (%)	1.033	0.912 – 1.170	n.s.

†Corrected for sex, age, BMI, plateletcount, fibrinogen- and FVIII:C-levels.

Outcome	Predictor†		Odds Ratio	95% CI	p
BUC	Thrombin generation	Lagtime (min)	1.109	0.813 – 1.511	n.s.
		ETP (nM*min)	0.999	0.998 – 1.000	n.s.
		Peakheight (nM)	0.987	0.976 – 0.997	0.016
		Time to peak (min)	1.252	0.969 – 1.618	n.s.

†Corrected for sex, age, BMI, fibrinogen- and FXIII:C-levels.

Outcome	Predictor†		Odds Ratio	95% CI	p
BUC	Plasma clot lysis	CLT (min)	0.997	0.957 – 1.039	n.s.
		CLT PCI+ (min)	0.976	0.899 – 1.059	n.s.

†Corrected for sex, age, BMI, fibrinogen- and FXIII:C-levels.

Abbreviations: BUC: bleeding of unknown cause; MBD: mild bleeding disorder; CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness; ML: maximum lysis; ETP: endogenous thrombin potential; CLT: clot lysis time; PCI: potato carboxypeptidase inhibitor; n.s.: non-significant.

Supplemental table 4. Haemostatic variables based on bleeding score

	Normal bleeding score (n = 137)	Abnormal† bleeding score (n = 117)	p
Hemoglobin, mmol/L	8.2 [7.7 – 8.7]	8.3 [8.0 – 8.9]	0.01
Plateletcount, 10^9/L	266 [230 – 301]	262 [219 – 313]	n.s.
PT, sec	11.9 [11.5 – 12.6]	11.7 [11.9 – 12.5]	n.s.
APTT, sec	25 [23 – 26]	25 [24 – 26]	n.s.
PFA, sec	137 [116 – 159]	139 [119 – 162]	n.s.
Fibrinogen, g/L	2.8 [2.4 – 3.4]	2.7 [2.4 – 3.3]	n.s.
VWF:Act, U/ml	0.93 [0.75 – 1.25]	0.85 [0.70 – 1.12]	0.03
FVIII:C, U/ml	1.22 [1.05 – 1.53]	1.16 [1.00 – 1.29]	0.00
FVII:C, U/ml	0.94 [0.79 – 1.11]	0.90 [0.72 – 1.17]	n.s.
FIX:C, U/ml	1.02 [0.96 – 1.14]	1.05 [0.94 – 1.15]	n.s.
FXI:C, U/ml	1.06 [0.97 – 1.15]	1.03 [0.96 – 1.14]	n.s.
FXIII:C, U/ml	1.28 [1.11 – 1.38]	1.23 [1.06 – 1.36]	n.s.
Alpha-2 antiplasmin, U/ml	1.11 [1.02 – 1.18]	1.15 [1.07 – 1.20]	n.s.

†Abnormal bleeding scores: ≥6 for female, ≥4 for male, ≥3 for adolescents.

Abbreviations: PT: prothrombin time; APTT: activated partial thromboplastin time; PFA: platelet function analyzer; VWF:Ag: von Willebrand factor antigen; VWF:Act: von Willebrand factor activity; VWF:CB: von Willebrand factor collagen binding; FVIII:C: factor VIII activity; FVII:C factor VII activity; FIX:C: factor IX activity; FXI:C: factor XI activity; FXIII:C; factor XIII activity; n.s.: non-significant.

Supplemental table 5. Rotational thromboelastometry, thrombin generation and plasma clot lysis variables based on bleeding score

		Normal bleeding score (n = 137)	Abnormal† bleeding score (n = 117)	p
ROTEM – EXTEM	CT (min)	65 [58 – 72]	67 [63 – 73]	0.048
	CFT (min)	73 [63 – 88]	77 [63 – 94]	n.s.
	MCF (mm)	66 [62 – 69]	65 [62 – 67]	n.s.
	ML (%)	7 [5 – 9]	8 [6 – 9]	n.s.
ROTEM – INTEM	CT (min)	187 [171 – 202]	184 [169 – 200]	n.s.
	CFT (min)	71 [62 – 79]	70 [62 – 86]	n.s.
	MCF (mm)	62 [59 – 65]	62 [58 – 65]	n.s.
	ML (%)	8 [6 – 12]	9 [6 – 11]	n.s.
ROTEM – FIBTEM	CT (min)	60 [55 – 68]	63 [57 – 72]	n.s.
	MCF (mm)	16 [13 – 19]	15 [11 – 18]	0.04
	ML (%)	2 [0 – 6]	2 [0 – 5]	n.s.
Thrombin generation	Lagtime	7.3 [6.2 – 8.6]	7.4 [6.6 – 8.6]	n.s.
	ETP	1223 [923 – 1516]	1055 [828 – 1363]	0.046
	Peak height	126.9 [90.7 – 166.1]	105.9 [83.1 – 147.5]	n.s.
	Time to peak	12.9 [11.7 – 14.2]	13.3 [12.0 – 14.6]	n.s.
Clot lysis time	PCI-	57.6 [54.0 – 62.9]	59.6 [54.3 – 65.4]	n.s.
	PCI+	39.8 [36.9 – 44.2]	40.7 [37.3 – 45.3]	n.s.

†Abnormal bleeding scores: ≥6 for female, ≥4 for male, ≥3 for adolescents.

Abbreviations: CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness; ML: maximum lysis; ETP: endogenous thrombin potential; CLT: clot lysis time; PCI: potato carboxypeptidase inhibitor; n.s.: non-significant.

Supplemental table 6. Reference values for rotational thromboelastometry, thrombin generation and plasma clot lysis time calculated based on 76 healthy controls

ROTEM	EXTEM	INTEM	FIBTEM	Thrombin generation		Plasma clot lysis assay	
CT, sec	47 – 185	127 – 242	46 – 239	Lag time, min	4.29 – 10.59	Clot lysis time, min	43.33 – 116.80
CFT, sec	47 – 124	50 – 125		ETP, nM*min	528.69 – 2345.73	Clot lysis time PCI+, min	30.73 – 82.95
α	65 – 81	67 – 79	52 – 80	Peak height, nM	43.86 – 477.09		
A10, mm	39 – 69	44 – 65	8 – 24	Time to peak, min	6.56 – 17.07		
A20, mm	49 – 73	51 – 69	8 – 25	Velocity Index, nM/min	6.12 – 232.95		
MCF, mm	55 – 73	46 – 69	8 – 25	Start Tail, min	23.59 – 38.95		
ML, %	1 – 62	1 – 72	0 – 15				

Reference ranges for ROTEM, thrombin generation and plasma clot lysis time were calculated with the Reference Value Advisor Software (v2.1) which closely follows the CLSI guideline [19,20] based on 76 healthy controls. Abbreviations: CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness; ML: maximum lysis; ETP: endogenous thrombin potential; CLT: clot lysis time; PCI: potato carboxypeptidase inhibitor

CHAPTER 4

FIBTEM Clot Firmness Parameters Correlate Well with the Fibrinogen Concentration Measured by the Clauss Assay in Patients and Healthy Subjects

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SUMMARY

The Clauss assay is the assay most often used for measuring plasma fibrinogen levels. However, the FIBTEM-assay, determined using thromboelastometry (ROTEM) can also be used to estimate fibrinogen levels. A major advantage of the FIBTEM is that it can provide information about fibrinogen levels within minutes, while the Clauss assay needs 30-60 minutes before results are available. The aim of this study was to investigate the correlation between fibrinogen levels measured by the Clauss assay and results from the FIBTEM-assay. We included 111 patients ≥18 years for whom both ROTEM analyses and a fibrinogen measurement using the Clauss assay were available. In addition, ROTEM and Clauss measurements from 75 healthy subjects were included. Spearman correlation was used to determine the association between results of both assays. The patients included were mostly patients with major trauma or undergoing large surgery (e.g. cardiac surgery or liver transplantation). Strong correlations were found between FIBTEM clot firmness parameters and fibrinogen levels measured by the Clauss assay in patients (Spearman's correlation coefficients (r_s) above 0.80 ($p<0.001$) for all subgroups) and healthy subjects ($r_s=0.66$, $p<0.001$). The correlation between early FIBTEM parameters (clot firmness at 5 or 10 minutes) and the maximum clot firmness was almost perfect (r_s above 0.96). Also, the correlation between the α-angle and FIBTEM parameters was strong (r_s above 0.7). In conclusion, strong correlations were found between early FIBTEM parameters and fibrinogen levels.

INTRODUCTION

During acute settings accompanied by major blood loss (e.g. major trauma or complicated surgical procedures), it is important for clinicians that fibrinogen concentrations are available as quickly as possible, in order to guide adequate management. The risk of bleeding is increased in individuals when fibrinogen levels decrease during trauma or surgery and it is recommended to maintain them above 1.5 g/L [1].

For the most commonly used fibrinogen assay, the Clauss assay, it takes 30-60 minutes before results are known in a diagnostic laboratory [2]. Another method that is regularly used in acute settings to rapidly estimate fibrinogen concentration is rotational thromboelastometry (ROTEM). A specific test of the ROTEM, the FIBTEM, provides information about the extrinsic pathway of coagulation, while eliminating the role of platelets. Therefore, information obtained with this assay gives an estimate of the contribution of fibrinogen to coagulation. Different parameters can be obtained from the FIBTEM test, of which the amplitude (or clot firmness) at five minutes (A5) or 10 minutes (A10) and the maximum clot firmness (MCF) are most used for estimating fibrinogen levels.

It is suggested that the A5 can already provide relevant information about the functional fibrinogen concentration [3]. In addition, the α-angle might be a good indicator for the value of the A5 or A10 [4]. Differences in the underlying mechanism of the Clauss assay and FIBTEM test can give discrepant results, especially in patients with dysfibrinogenemia or low levels of coagulation factors [5,6]. This is specifically relevant in trauma patients and patients undergoing large surgeries. Furthermore, in healthy individuals, heterogeneity in fibrinogen can potentially affect the results of both assays, resulting in discrepancies [7]. The correlation between the FIBTEM and Clauss assay has been investigated before, however only in selected groups of patients [8], and no information is available for this correlation in healthy individuals.

Therefore, the aim of this study was to determine correlations between fibrinogen concentration measured by the Clauss assay and FIBTEM clot firmness parameters in different patient groups in a real-life hospital setting and in healthy individuals.

METHODS

Patients

Data from all patients aged ≥18 years for whom both ROTEM and Clauss assays were ordered as part of routine care in May or June 2019 in the Erasmus Medical Center Rotterdam were collected retrospectively for this study. Patients for whom no results were available for the ROTEM, Clauss assay or APTT were excluded; there were no other in- or exclusion criteria. Included patients were divided into the following groups: major bleeding or other trauma, liver transplantation or other liver surgery, cardiac surgery (mainly procedures involving

heart valves or aorta) and other (mainly other surgical procedures). The following parameters were obtained from patients laboratory results: prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen concentration (Clauss assay) and ROTEM results. Only one measurement of each patient was included, namely the first measurement in which the APTT was below 100 seconds, to exclude results strongly influenced by heparin. Based on the retrospective nature of this study, this study was not subject to the Medical Research Involving Human Subjects Act and a waiver for informed consent was granted for the patient group (MEC-2020-0507). As part of the Crescendo study (Clinical Relevance and Significance of New Diagnostic Options in patients with Unexplained Bleeding) healthy individuals were recruited between July 2016 and March 2018 among employees and students of the Erasmus MC University Medical Center [9]. The Crescendo study was subject to the Medical Research Involving Human Subjects Act and approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2016-218). Written consent was obtained from each healthy participant. All healthy volunteers with results of both the ROTEM and Clauss assay (n=75) available were included in the current study.

Fibrinogen Assays

The Clauss assay was performed on a fully-automated coagulation analyser (Sysmex CS-5100 system, Siemens Healthcare Diagnostics, Breda, the Netherlands). FIBTEM measurements were performed on the ROTEM® Delta device, according to the manufacturer's instructions (Werfen, Barcelona, Spain). The following ROTEM parameters were analysed: clot firmness at 5 or 10 minutes (A5 and A10, respectively), maximum clot firmness (MCF) and the α-angle.

Statistical Analysis

We used descriptive statistics to summarize baseline characteristics of the study group. Because of a skewed distribution, all data are presented as median with interquartile range (IQR). Correlations between the Clauss assay and ROTEM parameters were tested by non-parametric analyses, determining Spearman's correlation coefficients. Kappa statistics was done to test the agreement in classification in three groups (low, normal, high) between the two assays. Receiver operating characteristic (ROC) curves were used to determine the best cut-off value of early FIBTEM parameters to predict low fibrinogen levels (below 1.5 g/L). All tests were two-tailed and a p-value below 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics 25.

RESULTS

A total of 111 patients of whom both ROTEM and Clauss assay results were available were included in this study, in addition to 75 healthy subjects. The median [IQR] age was 60.0 [49.0

-69.0] for the patients and 32.0 [26.0-46.0] for the healthy subjects. 38% of the patients was female, while in the healthy subjects the fraction of women was 85%. The majority of patients of whom ROTEM measurements were available, were patients undergoing cardiac surgery (40%) or patients that experienced bleedings or other trauma (31%). Twelve percent of patients had a liver transplantation or liver surgery and 18% of patients were classified as 'other'. In this last group, mainly patients undergoing surgical procedures, other than cardiac or liver surgery (for example laparotomy), were included. In the patient subgroups, the fraction of women was between 16% and 65% (Table 1). As expected, PT and APTT values were significantly higher in the patient groups compared to healthy subjects.

Table 1. Study group characteristics

Characteristic	Healthy subjects (n=75)	Bleeding/ rauma (n=34)	Liver surgery (n=13)	Cardiac surgery (n=44)	Other (n=20)
Age (years)	32.0 [26.0-46.0]	56.0 [33.3-65.0]	52.0 [49.0-66.0]	67.5 [56.3-75.0]	55.0 [42.5-65.5]
Sex (women)	64 (85%)	16 (47%)	6 (46%)	7 (16%)	13 (65%)
PT (s)	11.9 [11.5-12.6]	14.1 [12.3-17.9]	15.1 [12.9-18.0]	15.2 [13.5-16.8]	13.8 [11.7-17.5]
APTT (s)	25.0 [23.0-26.0]	27.5 [24.0-33.3]	30.0 [29.0-33.5]	29.0 [26.0-35.8]	27.0 [23.3-38.3]
Fibrinogen[a] (g/L)	2.7 [2.3-3.4]	1.9 [1.5-2.6]	2.4 [1.9-3.3]	1.9 [1.5-2.2]	3.2 [1.7-4.3]
FIBTEM α-angle	69.0 [64.0-74.0]	69.5 [62.8-75.8]	73.0 [71.0-80.0]	71.0 [67.5-76.5]	74.0 [64.0-77.0]
FIBTEM A5 (mm)	ND	11.0 [8.3-13.5]	12.0 [9.0-18.5]	10.5 [7.3-13.0]	14.5 [8.3-19.0]
FIBTEM A10 (mm)	15.0 [12.0-17.0]	11.5 [8.5-14.5]	14.0 [10.0-20.0]	12.0 [9.0-14.0]	15.5 [8.5-22.8]
FIBTEM MCF (mm)	15.0 [12.0-18.0]	12.5 [9.5-16.3]	16.0 [11.0-22.5]	13.0 [9.3-15.0]	17.5 [9.8-24.5]

Data are presented as median [interquartile range] or absolute number (%).
Abbreviations: A5, amplitude (clot firmness) at 5 minutes; A10, amplitude at 10 minutes; APTT, activated partial thromboplastin time; MCF, maximum clot firmness; ND, not determined; PT, prothrombin time.
[a] Patients with major blood loss or very low levels of fibrinogen might have received fibrinogen concentrate, which could have had an impact on these fibrinogen measurements

The correlation between the clot firmness at 5 or 10 minutes (A5 and A10) and the maximum clot firmness (MCF) of the FIBTEM was almost perfect in all patient subgroups and healthy individuals (r_s above 0.96, $p<0.001$) (Figure 1 and Supplementary Table I). In addition, the α-angle and the clot firmness parameters were strongly correlated in all subgroups (Table 2).

Figure 2 and Table 2 show the correlations and Spearman's correlation coefficients for fibrinogen levels measured by the Clauss assay and the FIBTEM clot firmness parameters. In both the patients groups as well as healthy individuals, strong correlations between the Clauss assay and FIBTEM were found: r_s above 0.80, $p<0.001$ for all patient subgroups and $r_s=0.66$, $p<0.001$ for healthy subjects. The correlation in the healthy individuals is somewhat lower, probably because of the smaller range of the fibrinogen levels.

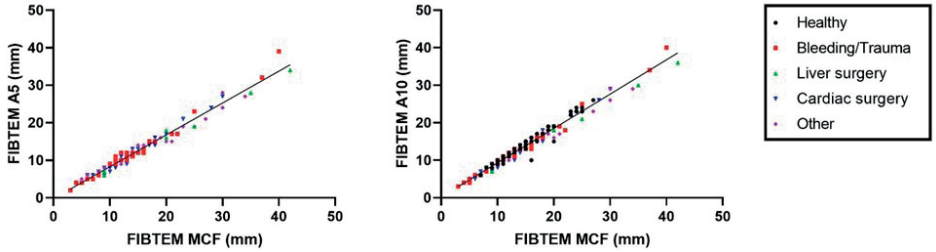

Figure 1. Correlation between the maximum clot firmness (MCF) of the FIBTEM assay and early FIBTEM parameters (clot firmness at 5 (A5) or 10 (A10) minutes).

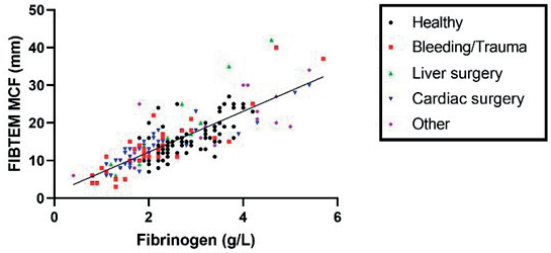

Figure 2. Correlation between fibrinogen concentrations measured by the Clauss assay and maximum clot firmness (MCF) of the FIBTEM assay.

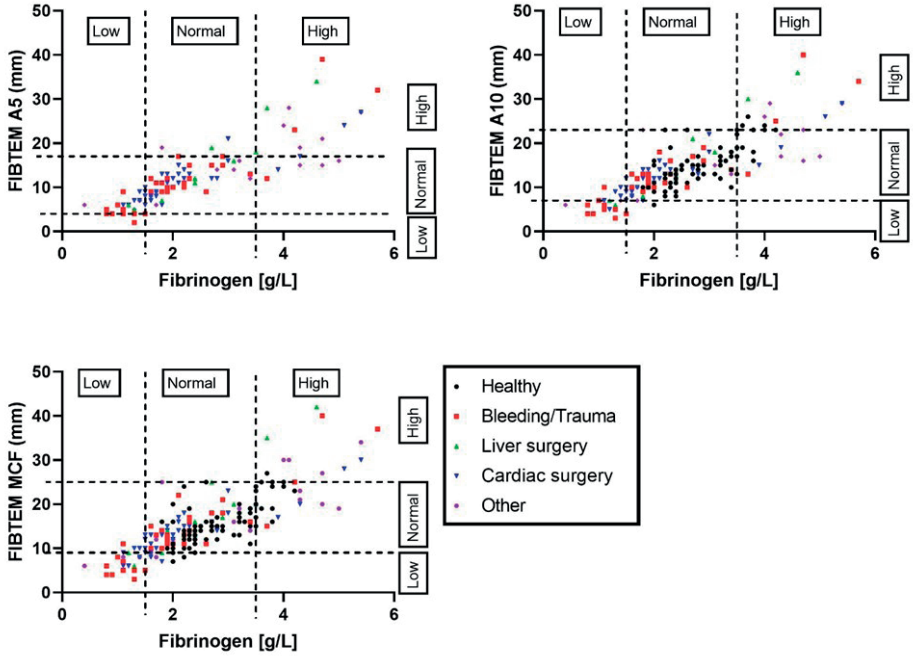

Figure 3. Agreement between classification in low, normal and high levels according to the Clauss assay and FIBTEM parameters clot firmness at 5 (A5) or 10 (A10) minutes or maximum clot firmness (MCF).

Table 2. Correlation between fibrinogen level (Clauss assay) or the FIBTEM α-angle and FIBTEM clot firmness parameters

FIBTEM parameter	Healthy (n=75)	Bleeding/Trauma (n=34)	Liver surgery (n=13)	Cardiac surgery (n=44)	Other (n=20)
A5 vs Clauss		0.846 [0.683-0.918]	0.939 [0.741-0.999]	0.899 [0.805-0.946]	0.808 [0.518-0.943]
A10 vs Clauss	0.697 [0.527-0.829]	0.839 [0.669-0.917]	0.941 [0.755-0.999]	0.899 [0.800-0.946]	0.803 [0.446-0.948]
MCF vs Clauss	0.657 [0.457-0.802]	0.848 [0.678-0.923]	0.935 [0.750-0.991]	0.892 [0.794-0.939]	0.821 [0.496-0.952]
A5 vs α-angle		0.912 [0.788-0.958]	0.917 [0.630-0.998]	0.850 [0.655-0.947]	0.850 [0.568-0.972]
A10 vs α-angle	0.722 [0.575-0.831]	0.900 [0.759-0.952]	0.938 [0.687-1.000]	0.842 [0.634-0.943]	0.837 [0.502-0.978]
0MCF vs α-angle	0.718 [0.564-0.829]	0.841 [0.613-0.937]	0.926 [0.700-1.000]	0.777 [0.528-0.900]	0.819 [0.398-0.900]

Data are presented as r_s [95% confidence interval]. All p-values <0.001. r_s: Spearman's correlation coefficient, p-values by Spearman's rank test

Table 3. Agreement between the Clauss assay and FIBTEM parameters in classification in low, normal or high fibrinogen

FIBTEM parameter (normal range)	Healthy subjects (n=75)	Bleeding/Trauma (n=34)	Liver surgery (n=13)	Cardiac surgery (n=44)	Other (n=20)
A5 (4-17 mm)		0.693 [0.452-0.934]	0.350 [-0.107-0.807]	0.138 [-0.066-0.342]	0.381 [0.063-0.699]
A10 (7-23 mm)	0.388 [0.711-0.065]	0.885 [0.730-1.040]	1.000 [1.000-1.000]	0.507 [0.256-0.758]	0.514 [0.204-0.824]
MCF (9-25 mm)	0.085 [-0.140-0.310]	0.822 [0.634-1.010]	0.851 [0.571-1.131]	0.536 [0.287-0.785]	0.528 [0.218-0.838]

Data are presented as kappa statistics with [95% confidence interval] of the agreement in classification in 3 groups according to fibrinogen level measured by the Clauss assay (low: ≤1.5; normal: 1.5-3.5 or high: >3.5 g/L) and FIBTEM parameters (low: below normal range, normal: within normal range, high: above normal range).

In addition, the agreement between the two assays in classifying fibrinogen levels in low, normal and high was calculated (Table 3). The fibrinogen measurements were divided in three groups: ≤1.5 g/L, between 1.5 and 3.5 g/L and >3.5 g/L. In addition, the levels of the FIBTEM parameters were categorized in three groups (lower than or equal to the normal range, in the normal range, or above the normal range as provided by the manufacturer of the ROTEM instrument) (Figure 3). Especially for A10 and MCF, the agreement between the FIBTEM parameters and the Clauss assay was found to be strong (K above 0.5, p<0.001) (Table 3).

Finally, we determined the optimal cut-off values of the FIBTEM parameters to identify fibrinogen levels below 1.5 g/L, based on our study population instead of the general reference values provided by the manufacturer. This was only done for the 111 patients, since there were no healthy subjects with fibrinogen levels below 1.5 g/L. In total, 27 out of 111 patients (24.3%) had fibrinogen levels below 1.5 g/L. The optimal cut-off value was ≤9.5 for both A5 and A10 values and ≤10.5 for MCF (Table 4).

Table 4. Optimal cut-off values of FIBTEM parameters for fibrinogen levels ≤1.5g/L

FIBTEM parameter	Cut-off value	Sensitivity	Specificity
A5	9.5	96.3%	82.1%
A10	9.5	85.2%	90.5%
MCF	10.5	88.9%	88.1%

Cut-off values of FIBTEM parameters to predict fibrinogen levels below 1.5 g/L. Receiver operating characteristic (ROC) curves were made, after which the optimal cut-off values were determined using the Youden index.

DISCUSSION

We compared the results of fibrinogen levels measured by the Clauss assay with those of the FIBTEM assay and found high correlations in different patient groups and healthy individuals. We also found an almost perfect correlation between the MCF of the FIBTEM and the A5 or A10, which implicates that early parameters of the FIBTEM predict the final clot firmness. Finally, strong correlations were found between the α-angle and clot firmness parameters, implicating that faster clot formation also predicts higher clot firmness.

The correlation between the Clauss assay and the FIBTEM assay is strong for most patients (r_s above 0.80, p<0.001), however some individuals have discrepant values. This could have been caused by dysfibrinogenemia or low levels of coagulation factors caused by trauma or surgery. Previously, other studies have been performed to correlate FIBTEM measurements with fibrinogen levels measured by the Clauss assay in different patient groups [8]. In women with postpartum hemorrhage, moderate to good correlations have been found between the Clauss assay and FIBTEM parameters A5, A10 or MCF [10-12]. Also in children [13], trauma patients [14-16] and patients undergoing liver transplantation [17-19] or cardiac surgery [20,21], moderate to

good correlations have been found between fibrinogen concentrations and the different FIBTEM parameters. A potential confounder in the patients undergoing cardiac surgery in our study is heparinization, which might have interfered with the FIBTEM measurements. However, a heparin inhibitor is present in the FIBTEM measurement, neutralizing high heparin concentrations up to 1 U/ml. To exclude samples with heparin concentrations above 1 U/ml, no measurements for which APTT results were above 100 seconds were included in the analyses. In addition, we do not observe a weaker correlation in patients undergoing cardiac surgery compared to the other subgroups, suggesting heparin did not influence the results. A strength of our study was that we retrospectively compared the results of both assays measured during normal clinical settings instead of selecting patients. This shows that the results are applicable to a wide range of patients. In addition, we included a large healthy population to compare the Clauss assay with FIBTEM parameters, which, to our knowledge, has not been reported before. The correlation between fibrinogen levels and FIBTEM parameters was slightly lower in healthy individuals compared to the patients. This is most likely caused by the much smaller range of fibrinogen levels in the healthy individuals.

When both assays were used to classify patients in low, normal or high levels of fibrinogen in this study, especially strong agreement was found in patients with bleedings or other trauma and patients undergoing liver transplantation or surgery. For patients undergoing cardiac surgery or other surgeries, the agreement was somewhat lower, which could have been due to the low number of patients in these groups. In addition, the reference values based on the manufacturer of the ROTEM instrument might not have been the best cut-off values to classify patients in low, normal or high fibrinogen levels, since the normal values potentially differ between different laboratories. Therefore, we determined the optimal cut-off values of the FIBTEM parameters, based on our study group, to predict whether fibrinogen levels are below 1.5 g/L. Multiple studies have looked at optimal threshold values of the early FIBTEM parameters A5 and A10 to quickly determine if fibrinogen levels are below a critical point. One study found a similar threshold for A5 as we did (9.5 mm) to determine fibrinogen levels below 1.5 g/L in trauma patients [15], while other studies determined lower threshold levels (5, 6 or 7 mm) [12,20,22,23]. For A10, only one study in patients undergoing cardiac surgery determined the optimal cut-off value, which was similar to ours [24]. It is important to work with the optimal cut-off values, because this prevents unnecessary supplementation of fibrinogen, while the risk of bleeding is reduced to a minimum.

In the Erasmus Medical Center, according to the massive blood loss protocol, fibrinogen concentrate is given to patients when A10 values are ≤9 mm. If A10 values are ≤7 mm or ≤5 mm, increased amounts of fibrinogen concentrate are given. The values currently used to guide transfusion of fibrinogen concentrate correspond well to the optimal cut-off value found in this study: 9.5 for fibrinogen levels below 1.5. As described above, the reported optimal cut-off values for FIBTEM parameters to determine low fibrinogen levels are quite variable across different studies. This might partially be caused by variation in assays and

reagents per laboratory, and the type of ROTEM used contributes to the variation [11]. It is therefore of great importance for each laboratory to work with reference values specific for the device used.

A limitation of our study is the retrospective nature, which may have introduced bias and increases the risk of statistical errors. However, a selection bias was minimized by including all patients for which ROTEM and Clauss measurements were ordered in May and June 2019. Therefore, this is a good representation of patient population for which ROTEM measurements are needed and these results are relevant. In addition, results from these measurements are not very likely to be wrongly recalled, since the raw data of these tests are available in the patient laboratory results. Another limitation is the limited number of subject with very low or very high fibrinogen levels. However, it is important to realize that this study was performed in a real-life hospital setting; there was no selection of samples based on the fibrinogen level. In addition, the healthy individuals were more often female and on average much younger than the patients included in this study, which makes the groups less comparable to each other. However, the aim of this research was not to compare these groups, but to investigate the correlation between the two assays in both groups. Finally, fibrinogen concentrate might have been given to patients included in this study, especially after major blood loss or during large surgeries. We believe this does not have consequences for our results, since this will both affect the Clauss assay and ROTEM measurement.

In conclusion, early FIBTEM clot firmness parameters correlate well with final clot firmness as measured by the FIBTEM assay and to fibrinogen concentration as measured by the Clauss assay. This means that early FIBTEM parameters as well as the MCF might be used to evaluate fibrinogen concentrations, thus saving time in emergency situations.

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Supplementary Table 1. Correlation between FIBTEM clot firmness parameters

FIBTEM parameter	Healthy (n=75)	Bleeding/Trauma (n=34)	Liver surgery (n=13)	Cardiac surgery (n=44)	Other (n=20)
A5 vs MCF		0.978 [0.934-0.993]	0.993 [0.961-1.000]	0.965 [0.932-0.982]	0.983 [0.903-0.997]
A10 vs MCF	0.961 [0.912-0.990]	0.990 [0.970-0.996]	0.997 [0.978-1.000]	0.971 [0.939-0.986]	0.991 [0.946-1.000]

Data are presented as r_s [95% confidence interval]. All p-values <0.001

PART II

Female specific health issues in Haemostasis and Thrombosis

CHAPTER 5

Severe postpartum haemorrhage as first presenting symptom of an inherited bleeding disorder

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ABSTRACT

Introduction Postpartum haemorrhage (PPH) is the major cause of maternal death worldwide. Haemostatic abnormalities are independently associated with a significantly increased risk for severe PPH. In this study, the value of haemostatic evaluation in women with severe PPH was explored.

Aim To investigate the occurrence of previously unknown inherited bleeding disorders in women with severe PPH.

Methods Women with severe PPH (blood loss of ≥2000 mL) between 2011 – 2017, referred to the haematology outpatient clinic for haemostatic evaluation, were retrospectively included. A bleeding disorder was diagnosed based on (inter)national guidelines, or when having a clear bleeding phenotype, not fulfilling any diagnostic criteria or laboratory abnormalities, this being classified as Bleeding of Unknown Cause (BUC). Logistic regression was used to model the association between diagnosis and obstetrical causes and risk factors for PPH.

Results In total, 85 women with PPH were included. In 23% (n=16) a mild bleeding disorder was diagnosed, including low Von Willebrand factor (Low VWF 8/16), platelet function disorders (PFD 5/16), BUC (2/16) and Von Willebrand Disease type 1 (1/16). No significant associations were found between obstetrical causes or risk factors for PPH and the presence of a bleeding disorder.

Conclusion In 23% of women with severe PPH a mild bleeding disorder was diagnosed, independent of obstetrical causes or risk factors for PPH. This implies that severe PPH can be the first clinical symptom of an inherited bleeding disorder. Therefore, to optimize clinical management, haemostatic evaluation after severe PPH is recommended.

INTRODUCTION

Postpartum haemorrhage (PPH) is still the major cause of maternal death worldwide, and the prevalence of PPH is steadily increasing in many high-resource countries[1,2]. Primary PPH is traditionally defined as 500 ml blood loss or more within 24 hours after delivery, independent of the mode of delivery[3,4]. PPH can further be classified as minor (500 – 1000 mL) or major (> 1000 mL) PPH[5], with major PPH being subdivided in moderate (1001 – 2000 mL) and severe (> 2000 mL) PPH[6,7,8]. The most frequent obstetrical causes of PPH are uterine atony, (partially) retained placenta, or perineal trauma (episiotomy and / or lacerations). A history of PPH, advanced maternal age, preeclampsia, macrosomia and multiple gestation are known risk factors for PPH[9,10]. However, the cause may be multifactorial and additional factors may remain unidentified in a significant number of cases.

It is well known that pre-existing coagulation disorders are a risk factor for PPH[9,11]. Whereas rare but severe inherited bleeding disorders are diagnosed early in life due to the severity of bleeding, mild bleeding disorders can remain undiagnosed until a haemostatic challenge (e.g. delivery) occurs[12]. Even mild haemostatic abnormalities, however, are independently associated with a significantly increased risk for severe PPH, which include decreased levels of fibrinogen, and low von Willebrand factor (VWF) levels[13].

Within this study, we explored the value of haemostatic evaluation after deliveries with severe PPH in women without a previously diagnosed bleeding disorder. The aim of our study was to investigate the presence of bleeding disorders in women with severe PPH.

MATERIALS AND METHODS

Study design and patients

A cohort study was conducted, in which all women who were routinely referred to the haematology outpatient clinic for haemostatic evaluation after a delivery complicated by severe PPH between 2011 and 2017, were included. Severe PPH was defined as blood loss of ≥2000 mL within 24 hours after delivery, irrespective of mode of delivery. Women with blood loss of < 2000 mL within 24 hours after delivery and women previously diagnosed with a bleeding disorder were excluded. This study was not subject to the Medical Research Involving Human Subjects Act and approved by the Medical Ethics Committee of the Erasmus University Medical Centre Rotterdam.

Data collection

Medical records were reviewed to obtain details of the deliveries and estimated blood loss. The amount of blood loss was visual estimated until a blood loss of 500 mL. In case of more than 500 mL blood loss, the amount was estimated by measuring the volume of the blood

lost and by weighing the drapes, as routinely is performed. In addition, data on the presence of obstetrical risk factors, as documented in literature[9], administration of uterotonic agents, and use of antifibrinolytic agents were collected.

During evaluation of the included women at the outpatient clinic, a bleeding assessment tool (BAT) was used to objectify the bleeding tendency. A BAT is a standardized, quantitative tool that can translate the severities of a range of bleeding symptoms into a final, summative bleeding score. The BATs used in our clinic were either the Condensed MCMDM-1 VWD bleeding questionnaire (2011 – 2015), or the ISTH-BAT (2015 – present)[14,15]. The cut-off value for an abnormal bleeding score using the Condensed MCMDM-1 VWD bleeding questionnaire is ≥4[14]. For the ISTH-BAT, cut-off value for an abnormal bleeding score is ≥6 in female adults[15].

At time of haemostatic evaluation at the outpatient clinic, blood was taken. Haemostatic variables were preferably measured more than three months after delivery because of expected complete normalization of the haemostatic system at that time. Blood sampling by venipuncture was performed using the Vacutainer system (Becton Dickinson) containing sodium citrate (final concentration 0.109 mol/L) or EDTA (1.8mg/ml, Plymouth). Routine coagulation tests aPTT (Actin FS), PT (Thromborel S) and fibrinogen (Thrombin Reagent) were measured on a Sysmex CS5100 (Siemens Healthcare Diagnostics B.V.). Collagen-ADP (C-ADP) and collagen-epinephrine (C-Epi) cartridges were used to measure closure times (CT, seconds) on the PFA-200 (Siemens). Light Transmission Aggregometry (LTA) was performed on a Chrono-Log aggregometer 490 (Stago Benelux B.V.). Von Willebrand factor antigen (VWF:Ag) levels were determined with an in-house ELISA assay, using polyclonal rabbit antihuman VWF antibodies (DakoCytomation) for capturing and detecting. Von Willebrand factor collagen binding (VWF:CB) activity was measured by an in-house ELISA assay using bovine Achilles tendon collagen type I for capturing (Sigma-Aldrich) and polyclonal rabbit antihuman VWF antibodies (DakoCytomation) for detecting. Von Willebrand factor activity (VWF:GPIbM) was determined with the INNOVANCE VWF Ac assay (Siemens) on a Sysmex CS5100. FVIII and FIX coagulant activity (FVIII:C) was measured using one-stage clotting assays and derived from (the prolongation of) the clotting time (APTT) measured on the Sysmex CS-5100 (Siemens). FXIII activity was measured using the Berichrom® FXIII kit (Siemens) on the Sysmex CS5100 (Siemens). Alpha 2-antiplasmin level was measured using a chromogenic assay (Stachrom, Stago) on the Sysmex CS5100 (Siemens).

A bleeding disorder was confirmed if the identified laboratory abnormalities were in accordance with the definitions of a bleeding disorder stated in national and international guidelines[16-18]. A bleeding disorder was also considered confirmed, if criteria were met for low von Willebrand factor levels (low VWF, defined as VWF activity levels between 0.30-0.50 U/mL and ratio of FVIII:C to VWF:Ag > 0.6[16] outside pregnancy), or a platelet function disorder not otherwise specified (PFD NOS) (defined as abnormalities found using light transmission aggregation testing (LTA), not fitting the pattern of any known platelet function

disorder[18]). Finally, bleeding was considered as Bleeding of Unknown Cause (BUC) in case of a clear bleeding phenotype (based on the severity of the medical history and bleeding score calculated by the use of a BAT), not fulfilling any of the diagnostic criteria or laboratory abnormalities.

Statistical analysis

We used descriptive statistics to summarize baseline characteristic of the study group. In case of a skewed distribution, data are presented as median and interquartile range (IQR). In case of a normal distribution, data are presented as mean and standard deviation (SD). Categorical data are presented as numbers with percentages. Logistic regression was performed to model the association between diagnosis and obstetrical causes and risk factors for PPH, odds ratios (ORs) and 95% CI were calculated. A p-value of < 0.05 was considered statistically significant. All analyses were performed with SPSS version 21.0 (IBM, Armonk, NY, USA).

RESULTS

Between 2011 and 2017, 224 women had a delivery complicated by severe postpartum haemorrhage (≥2000 mL blood loss) in our center, based on the maternity database of the department of Obstetrics and Gynaecology. Of these 224 women, 85 women were referred for haemostatic evaluation and included in this study. Of these 85 women, 10 women visited the haematology outpatient clinic during a subsequent pregnancy, and 6 women were non-compliant to their referral for haemostatic evaluation (see figure 1). The included women had a median blood loss of 3000 mL (IQR 2500 – 4000 mL) and a median age of 33 years (IQR 30-35 years) at time of the index delivery. Of these women, 41/85 had a previous delivery. Of these deliveries, 13/41 (32%) were complicated by PPH (≥500 mL within 24 hours after delivery), with a median blood loss of 2200 mL (IQR 1500 – 2875 mL) (see table 1). No significant differences were found in baseline characteristics between women with and without a diagnosed bleeding disorder.

Of 79/85 women haemostatic variables were available. In 69/79 women haemostatic variables were measured outside a subsequent pregnancy. In the 10 women that were pregnant at time of haemostatic evaluation, no bleeding disorders were diagnosed. These women were excluded from the no BD group, as diagnoses could be missed based on the physiological increase in coagulation factors during pregnancy. In 16 of the 69 women (23%) a bleeding disorder was diagnosed. The presence of low VWF was most prevalent (8/16), and one woman was diagnosed with Von Willebrand Disease (VWD) Type 1. A platelet function disorder (PFD) was diagnosed in five women, and bleeding history was considered clinical relevant but of unknown cause (BUC) in two women (see figure 1). Following these

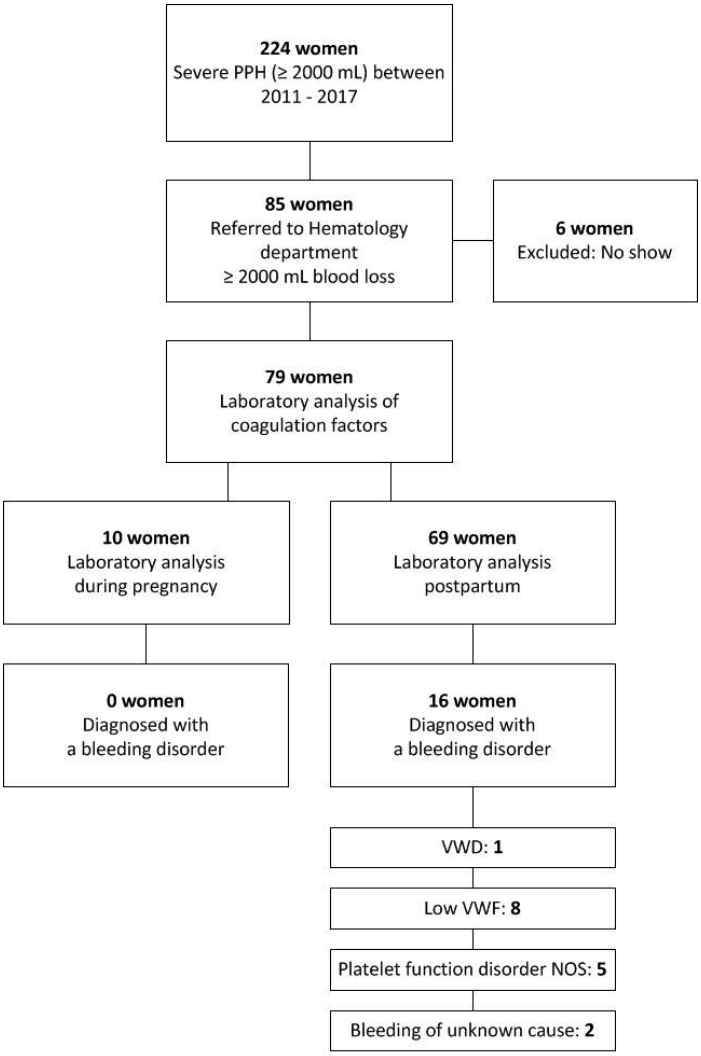

Figure 1. Flowchart of included women and found diagnoses

Flowchart of inclusion of women with PPH between 2011 and 2017, and number and percentage of diagnosed bleeding disorders. For statistical analysis, women that were pregnant at time of haemostatic evaluation (n=10) were excluded from the no BD group. Abbreviations: PPH: postpartum haemorrhage; VWD: Von Willebrand Disease; VWF: Von Willebrand Factor; NOS: Not otherwise specified. BD: bleeding disorder.

results, women with a diagnosed bleeding disorder had significantly lower median VWF:Act levels (0.61 U/ml [0.48 – 0.70 U/ml] versus 1.09 U/ml [0.83 – 1.34 U/ml], p=0.00) and significantly lower FVIII:C-levels (0.80 U/ml [0.69 – 1.15 U/ml] versus 1.26 U/ml [1.08 – 1.61 U/ml], p=0.00). Fibrinogen-levels did not differ significantly between women with and without a diagnosed bleeding disorder (2.4 g/L [2.3 – 2.9 g/L] versus 2.9 g/L [2.4 – 3.2 g/L], p=0.06), based on variables measured in the included non-pregnant women (see table 2).

Table 1. Study group characteristics

Characteristics	Women with severe PPH
N	85
Age at time of delivery (years)	33 [30 – 35]
Blood loss (mL)	3000 [2500 – 4000]
Vaginal delivery	64 (75%)
Elective C-section	13 (15%)
Emergency C-section	8 (10%)
Multiparity, n (%)	41 (48%)
Therapy†	
Erythrocyte transfusion	69 (91%)
Nr of packed cells (U)	2 [2 – 4]
Uterotonics	71 (99%)
Tranexamic acid	31 (44%)
PPH in medical history	13/41 (32%)
Blood loss (mL)	2200 [1500 – 2875]
Subsequent deliveries	25 (29%)
PPH in subsequent delivery	15/25 (60%)
Blood loss (mL)	2100 [1000 – 2500]
Positive family history of VWD†	1 (1%)

Data are presented as median (IQR) or number (%), as appropriate. †Based on available data. ‡ Based on available data from women ≥3 months postpartum. PPH: postpartum haemorrhage. VWD: Von Willebrand Disease. No significant differences were found between women with and without a diagnosed bleeding disorder.

Table 2. Characteristics of women with severe PPH: comparison of women with a diagnosed BD versus women with no diagnosis

Women with severe PPH	Diagnosed BD (n=16)	No BD (n=53)†	p
Bleeding score abnormal‡	31%	31%	ns
BS solely based on PPH	25%	47%	ns
Menorrhagia	56%	27%	0.034
Postsurgical bleeding	6%	7%	ns
Bleeding after tooth extraction	13%	0%	0.010
Blood type O	80%	33%	0.001
VWF:Ag (U/ml)§	0.61 [0.48 – 0.70]	1.09 [0.83 – 1.34]	0.000
FVIII:C (U/ml)§	0.80 [0.69 – 1.15]	1.26 [1.08 – 1.61]	0.000
Fibrinogen§	2.4 [2.3 – 2.9]	2.9 [2.4 – 3.2]	ns

Data are presented as median (IQR) or percentage (%), as appropriate. †Women that were pregnant at time of haemostatic evalution were excluded from the no BD group ‡Based on available data. ‡§ Based on available data from women ≥3 months postpartum. PPH: postpartum haemorrhage, BD: bleeding disorder, BS: bleeding score, VWF: Von Willebrand factor, FVIII:C: factor VIII-activity. Ns: non-significant. No significant differences were found between women with and without a diagnosis.

In 67/69 of the women a bleeding score was available. No difference was seen in the number of abnormal bleeding scores in women with and without a bleeding disorder, 31% (5/16) of patients with and 31% (16/51) of patients without a bleeding disorder had an abnormal bleeding score (p=0.99). PPH was the only bleeding symptom scored in 25% (4/16) of women with a bleeding disorder. Of women without a bleeding disorder, 47% (24/51) only scored PPH as bleeding symptom (p=0.12)(see figure 2). In the diagnosis group, 56% (9/16) of women scored on menorrhagia, compared to 28% (14/51) of the women without a diagnosis (p=0.04). Six percent of women with a diagnosis scored on postsurgical bleeding, compared to 6% of women without a diagnosis (p=0.97). For bleeding after tooth extraction, 13% of women with a diagnosis, compared to 0% of women without a diagnosis, scored 1 or more points on a BAT (p=0.01) (see table 2).

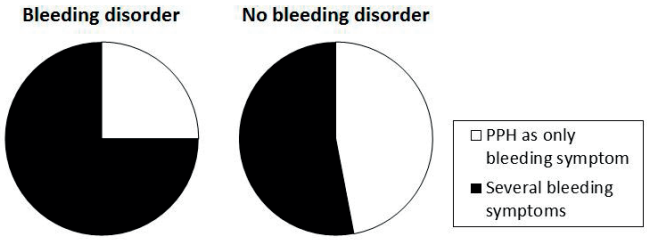

Figure 2. Percentage of women with and without a diagnosed bleeding disorder that only scored PPH as bleeding symptom on the ISTH-BAT

From 78/85 women complete data about obstetrical risk factors were available. In women with severe PPH, uterine atony (37/78, 47%), retained placenta (44/78, 56%) and preeclampsia (14/78, 18%) were frequently present (see table 3) and a median of 4 (IQR 3-5) obstetrical risk factors were present. After women that were pregnant at time of haemostatic evaluation were excluded from the no BD group, no differences were found in the number of obstetrical risk factors in women with a diagnosed bleeding disorder (n=16) (median 4, IQR 2-7) and in women without a bleeding disorder (n=53) (median 4, IQR 3-5)(p=0.71). Also, no significant differences were found in women with and without a diagnosed bleeding disorder with regard to obstetrical causes of PPH, e.g. uterine atony (OR 1.28, 95% CI 0.37 – 4.40), retained placenta (OR 0.77, 95% CI 0.20 – 2.95) or lacerations (OR 2.38, 95% CI 0.39 – 14.39) (see table 4).

Of the 85 patients, 99% received additional uterotonics during delivery. Only 44% of all women received tranexamic acid (TXA) (see table 1). Of the 13/85 women with PPH in their medical history, 6/13 (46%) received TXA. The administration of TXA was not associated with the year of delivery and no increase in administration of TXA during the years was seen.

Twenty-five of the 85 women with severe PPH had a subsequent delivery after haemostatic evaluation. Of these women, 15/25 women (60%) had recurrent PPH, with a median

Table 3. Number of present obstetrical causes and risk factors in women with PPH

Obstetrical risk factor for PPH	Women with severe PPH (n=78)†
Uterine atony	37/78 (47%)
Retained placenta	44/78 (56%)
Placenta previa	5/78 (6%)
Preeclampsia§	14/78 (18%)
Multiple gestation	7/78 (9%)
Fever	5/78 (6%)
Macrosomy > 4 kg	10/78 (13%)
Nulliparous	41/78 (53%)
Age >= 35 years	18/78 (23%)
Elective C-section	13/78 (17%)
Emergency C-section	9/78 (12%)
Induced labor‡	30/56 (54%)
Augmented labor‡	26/56 (46%)
Instrumental delivery‡	8/56 (14%)
Episiotomy‡	18/56 (32%)
Prolonged 3rd stage of labour‡	31/56 (57%)
Perineal lacerations‡	28/56 (48%)

Data presented as number of women with risk factor present (%) or median (IQR) as appropriate. †Of 78 women complete information about obstetrical risk factors during the delivery, complicated by severe postpartum haemorrhage, was available. ‡Based on the 56/78 women who had a vaginal delivery. PPH: postpartum haemorrhage. §As defined by the International Society for the study of Hypertension and Pregnancy (ISSHP) [24].

blood loss of 2100 mL (IQR 1000 – 2500 mL). Nine of these 15 (36%) women had recurrent severe PPH (see table 1). Of the 25 women with subsequent deliveries, 3/25 were diagnosed with a bleeding disorder after the first delivery complicated by severe PPH, namely VWD type 1, low VWF and a PFD. The woman diagnosed with VWD type 1 was treated according to protocol with Haemate P, but had excessive blood loss despite this treatment. The woman with low VWF had high and adequate VWF levels (VWF:Ag >1.50 U/mL) measured in the third trimester of pregnancy, and based on these levels according to protocol no precautions seemed necessary. Blood loss started a few hours after delivery, most probably based on a retained placenta, and at that time DDAVP was administered. Unfortunately, despite treatment there was excessive blood loss. The woman with a PFD was treated with DDAVP and TXA after a caesarean section, resulting in blood loss of 500 mL.

Table 4. Logistic regression analysis between presence and absence† of a diagnosed bleeding disorder and presence of important obstetrical causes and risk factors for PPH

Outcome	Predictor‡	Odds Ratio	95% CI	p
Diagnosis	Uterus atony	1.279	0.372 – 4.404	ns
	Retained placenta	0.774	0.203 – 2.953	ns
	Preeclampsia	0.742	0.132 – 4.182	ns
	Multiple gestation	0.830	0.074 – 9.270	ns
	Prolonged 3th stage of labor	0.666	0.161 – 2.759	ns
	Laceration	2.375	0.392 – 14.386	ns
	Episiotomy	0.340	0.060 – 1.917	ns
	Instrumental delivery	0.399	0.040 – 3.943	ns

†Women that were pregnant at time of haemostatic evalution were excluded from the no BD group ‡Corrected for age and mode of delivery. Ns: non-significant.

DISCUSSION

In this study, in 23% of women referred for haemostatic evaluation after severe primary PPH (≥ 2000 mL), a bleeding disorder was diagnosed. This high number implies that severe PPH can be a first clinical symptom of an inherited bleeding disorder. This finding is important for optimizing clinical management, in order to prevent bleeding complications during subsequent deliveries and interventions later in life. We did not find any significant differences in the presence of obstetrical causes and risk factors between patients with and without a diagnosed bleeding disorder. Therefore, an obstetrical cause or the presence of obstetrical risk factors in women with severe PPH does not automatically rule out an underlying haemostatic defect and a multifactorial cause of PPH is assumable.

It is already known that previous PPH results in a three times higher risk of recurrent PPH during a subsequent delivery[7,9,19]. In our severe PPH group, 60% of the subsequent deliveries was complicated by primary PPH (> 500 mL blood loss), with 36% of women having recurrent severe PPH (≥ 2000 mL blood loss). Less than half of the women with severe PPH received tranexamic acid at time of ongoing blood loss, even if they had a history of PPH.

Our study has several strengths and limitations. We included women with severe PPH, which we defined as blood loss of 2000 mL within 24 hours after delivery. Of these women, we collected both obstetrical and haematological data. In our analysis, we did not exclude women with obvious causes for PPH, such as uterine atony. Furthermore, we did an extensive haemostatic evaluation of the women with severe PPH, including platelet function testing. In total, 224 deliveries in our hospital were complicated by severe PPH between 2011 and 2017, of whom only 85 (38%) women were referred to our outpatient clinic for haemostatic evaluation. A selection bias may have occurred, when the gynaecologist considered referral only necessary when the excessive amount of blood loss occurred in combination with an atypical course of delivery and complications. Among the referred women, however, there were also

women with a clear obstetrical cause for PPH. We showed that the presence of obstetrical causes or risk factors did not differ between patients with and without a diagnosed bleeding disorder. Therefore, the presence of obstetrical causes and risk factors should not determine whether a patient is referred for haemostatic evaluation, specifically not in women with severe PPH. Another explanation of this possible selection bias is the non-compliance of women, shortly after a complicated and traumatic labor, not being aware of the importance of haemostatic evaluation three months postpartum. Another limitation is the evaluation of haemostatic abnormalities during pregnancy in 10 women. Available recommendations are not to test haemostatic parameters earlier than 8-12 weeks postpartum[20]. In the 10 women in whom no diagnosis was found during pregnancy, the physiological increase in Von Willebrand Factor caused by this pregnancy might mask a mild Von Willebrand Disease.

To our knowledge, only one study has previously investigated the value of primary PPH as predictor of inherited bleeding disorders[21]. In contrast to our study, this study included women with blood loss of ≥500 mL for spontaneous vaginal deliveries, ≥700 mL for instrumental deliveries and ≥1000 mL for caesarian sections, to define a group of women more likely to have abnormal coagulation. Possible platelet dysfunctions were not investigated. The authors concluded that PPH did not appear to be a strong predictor of inherited bleeding disorders, based on their finding that only 1/50 patients was diagnosed with an inherited bleeding disorder, which is much lower than 23% found in this study.

Assessing the bleeding history using standardized bleeding assessment tools is of high importance in women with severe PPH, as we found that 75% of women with a bleeding disorder had additional bleeding symptoms other than PPH. In addition, of the women with a diagnosis, women had significantly more menorrhagia compared to women without a diagnosis. Also bleeding after tooth extraction was more frequent in women with a diagnosed bleeding disorder, pointing to a possible disorder of primary hemostasis. Therefore, the presence of additional bleeding symptoms may be a reason for further extensive haemostatic evaluation. In line with our results, one study also mentioned the value of a Bleeding Assessment Tool, as the only woman diagnosed with a bleeding disorder was also the only patient who reported more than two additional bleeding symptoms[21]. Furthermore, we found that the majority of women was diagnosed with low VWF or mild VWD type 1 (9/16, 56%). This might indicate that, although there is a physiological increase in plasma VWF levels during pregnancy[9,22], some women with low VWF apparently still are at risk for PPH. This finding is in line with recently published data[23]. Based on these and our data, maintaining higher plasma VWF levels could be considered during the peripartum period, in addition to early administration of TXA. However, further studies will be needed to investigate the mechanisms underlying PPH in women with low VWF levels and optimal strategies for clinical management of these women. Also, it is recommended to also include a group of women without postpartum haemorrhage in a prospective cohort study, in order to investigate the incidence of low VWF levels in women without PPH[24]. This, also to prevent women of being

exposed to unnecessary treatment, as data available for DDAVP use in pregnancy are from a number of small trials and case studies[25], and the use of blood products should be avoided unless absolutely necessary

The elevated risk of recurrence of PPH, as confirmed in this study, highlights the need for a proactive attitude during the third stage of labor with early administration of uterotonics in women with a history of PPH. As been shown before, administration of tranexamic acid to women with PPH reduces deaths due to bleeding and laparotomy to control bleeding, with no evidence of any adverse effects or complications[26]. Therefore, additional haemostatic therapy, such as TXA, is highly recommended in women with (a history of) PPH.

CONCLUSION

In conclusion, based on this retrospective study in a subgroup of women referred for haemostatic evaluation, 23% of women with severe postpartum haemorrhage are diagnosed with a mild bleeding disorder. We therefore recommend a thorough bleeding history as first important step in identifying those more likely to have an underlying bleeding disorder, possible followed by routine screening for inherited bleeding disorders. In addition, because of the multifactorial nature of PPH, a proactive third stage of labour with a combination of early administration of uterotonics and additional haemostatic therapy, preferably tranexamic acid, especially in women with a history of PPH, is recommended. Based on haemostatic evaluation, management during future haemostatic challenges can further be guided, hereby-preventing bleeding complications later in life. However, prospective cohort studies are needed to confirm our findings and to further optimize clinical management for women with PPH.

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CHAPTER 6

Fibrin clot structure in major postpartum hemorrhage – a pilot study

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ABSTRACT

Introduction Postpartum hemorrhage (PPH) is a major cause of maternal death. Abnormal structure of the fibrin network in clots may contribute to bleeding, as clot structure affects clot characteristics, such as resistance to lysis and mechanical deformation. Therefore, the aim of this study was to investigate fibrin clot structure in women with and without major PPH.

Materials and Methods In this pilot study, we included 10 patients with major PPH (defined as blood loss ≥ 2000 mL) ≥3 months postpartum, and 5 controls with an uncomplicated delivery. Fibrin clot structure was studied by scanning electron microscopy and confocal laser scanning microscopy. Number of fibers, fiber diameter, fiber density, pore size and number of pores were analyzed using ImageJ software.

Results The median number of fibers and fiber diameter was 651 [597–701] and 0.130μm [0.127–0.136μm] in patients and 643 [611–677] (p=0.95) and 0.123μm [0.117–0.138μm] (p=0.39) in controls. The median number of thick (>0.133μm) and thin (≤0.133μm) fibers was 271 [237–294] and 337 [294–364] for patients and 199 [176–264] (p=0.11) and 400 [293–449] (p=0.39) for controls. The median fiber density, pore size and number of pores in patients and controls were 13.4% [10.9–17.3%] versus 15.2% [11.4–28.0%] (p=0.46), 10.63 μm^2 [4.04–24.60μm^2] versus 4.00μm^2 [2.35–14.98 μm^2] (p=0.33), and 638 [463–836] versus 752 [605–1010] (p=0.62).

Conclusion In this pilot study, several consistent trends towards differences in fibrin clot structure in women with and without major PPH were seen. Therefore, future studies investigating a disturbed fibrin clot structure as risk factor for major PPH, are recommended.

INTRODUCTION

Postpartum hemorrhage (PPH) is still one of the major causes of maternal death in the world, and the prevalence is steadily increasing in many high-resource countries[1,2], with an incidence of severe primary PPH of 4.5% in the general Dutch population[3]. Primary PPH is traditionally defined as the loss of 500 mL blood or more within 24 hours after delivery, independent of the mode of delivery[4,5], and major PPH is defined as blood loss of 1000 mL within 24 hours after delivery[6]. The most common causes of excessive bleeding postpartum are of gynecological origin, e.g. uterine atony, a (partially) retained placenta, or lower genital tract trauma. However, the cause may be multifactorial with pre-existing coagulation disorders[7,8], and even mild hemostatic abnormalities, being independently associated with an increased risk for PPH[9].

The fibrin clot structure is the major determinant of the mechanical stability and resistance to lysis of a clot[10]. A fibrin network can have thicker or thinner fibers, large or small pores and increased or decreased fiber density, and these parameters all affect the rate of fibrin dissolution, with clots with thinner fibers being more resistant to fibrinolysis than clots with thicker fibers, as well as being stiffer or more resistant to mechanical deformation[11]. For example, it has been previously shown that coagulation factor deficiencies cause clots with a reduced fiber density and relatively thick fibers[12-17]. In PPH, antifibrinolytic agents, such as tranexamic acid, have been proven to be of value during PPH[18], suggesting that bleeding might be caused by a higher fibrinolytic activity. Also, it is shown that even a mild decreased level of fibrinogen is associated with a significantly increased risk for major PPH[9]. Therefore, a disturbed fibrin clot structure might, at least in part, explain the bleeding risk in women who thus far have not been diagnosed with a bleeding disorder. Fibrin clot structure, however, is not examined in diagnostic testing.

We hypothesize that women who experienced postpartum hemorrhage have an altered fibrin clot structure compared to women without PPH, with thicker fibers, reduced fiber density and larger pore size, making the clot more susceptible to fibrinolysis and mechanical damage. To investigate this hypothesis, we performed a pilot study where we investigated the fibrin clot structure of women with and without major PPH.

MATERIALS AND METHODS

Patients and study design

As part of the Crescendo-study, patients with Bleeding of Unknown Cause (BUC) and healthy controls were recruited from July 2016 until March 2018 at our outpatient Hematology Clinic. For this pilot study, 10 women with major PPH (patients) and 5 women with a normal delivery (controls) were selected. Major PPH was defined as blood loss ≥ 2000 ml within

24 hours after delivery. This study was subject to the Medical Research Involving Human Subjects Act and approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam. All participants gave written informed consent.

Plasma preparation and clinical coagulation testing

Blood samples were acquired at least three months after delivery. Blood sampling was performed by venipuncture using the Vacutainer system (Becton Dickinson) containing sodium citrate (final concentration 0.109 mol/L). Citrated blood was centrifuged two times at 2000g for 10 minutes at room temperature, followed by 14000g for 10 minutes centrifugation of plasma at room temperature. Plasma samples were stored in aliquots at -80°C until analysis. Fibrinogen activity according to the Von Clauss'-method (Thrombin Reagent, Siemens), and factor FXIII (FXIII) activity using the Berichrom® FXIII kit (Siemens), were measured on a Sysmex CS5100 (Siemens Healthcare Diagnostics B.V.).

Routine coagulation tests aPTT (Actin FS) and PT (Thromborel S) were measured on a Sysmex CS5100 (Siemens Healthcare Diagnostics B.V.). Collagen-ADP (C-ADP) and collagen-epinephrine (C-Epi) cartridges were used to measure closure times (CT, seconds) on the PFA-200 (Siemens). Von Willebrand factor activity (VWF:GPIbM) was determined with the INNOVANCE VWF Ac assay (Siemens) on a Sysmex CS5100. FVIII coagulant activity (FVIII:C) was measured using one-stage clotting assays and derived from (the prolongation of) the clotting time (APTT) measured on the Sysmex CS-5100 (Siemens).

Fibrin structure analysis

Scanning Electron Microscopy

Clots for scanning electron microscopy were prepared as described previously[19]. Briefly, clot formation was initiated by the addition of thrombin and CaCl2 to PPP, with a final concentration of 0.5 U/ml Thrombin and 25 mM CaCl2. Clots were left to form at room temperature in a dark and moist atmosphere for 30 minutes. After polymerization, fibrin clots were washed three times with sodium cacodylate buffer and subsequently fixed in a 2% glutaraldehyde solution for 2 hours. The fixed clots were then stepwise dehydrated in 30, 50, 70, 90, and 95% ethanol solutions, and 3 times in 100% ethanol. The procedure was completed by chemical drying with hexamethyldisilazane (HMDS), and sputter coating with gold palladium. The clots were examined and photographed with a Quanta FEG250 FEI/Thermo Fisher Scientific scanning electron microscope (Hillsboro, OR, USA). In total, 8 images of every clot in randomly selected areas of the clot were acquired at 10,000x magnification. The number of fibers and fiber diameters were measured with image analysis software package ImageJ (1.52b, Wayne Rasband, National Institutes of Health, USA). As there is no clear definition of what can be considered as thick or thin fibers, we defined thick fibrin fibers as fibers with a diameter of > 0.133 μm and thin fibers as fibers with a diameter of ≤0.133 μm, based on a diameter of 0.133 μm being the mean fiber diameter of the total group. To exclude bias, the

micrographs were analyzed by an operator blind to the nature of the samples, e.g. patients versus control.

Confocal Laser Scanning Microscopy

Clots for confocal laser scanning microscopy were produced by incubating recalcified (25mM CaCl2, final) PPP with thrombin (0.5 U/ml, final) in glass chamber slides. The plasmas were spiked with trace AlexaFluor488-conjugated fibrinogen (4% of total fibrinogen, final) to visualize fibrin fibers, as described[15]. Clots were left to form in a dark and moist atmosphere for 1 h and then stored at 4°C overnight. Clots were scanned with a Zeiss LSM 880 confocal laser scanning microscope using a C-Apochromat 40x water immersion objective lens and a PMT detector. Optical Z stacks were acquired every 0.45 µm over 52 µm and then transformed by maximum projection into one image per scanned clot area. Three randomly selected fields of a surface of 141 x 141 micrometer area per clot were viewed. Fiber density and pore size were obtained by an in-house designed macro (Image J 1.52b), and quantified by summing individual sections to create Z-projections and thresholding to visualize fibers and / or pores and minimize noise. The area covered by pixels corresponding to the set threshold cutoff was determined.

Statistical analysis

The data were not normally distributed, so we used non-parametric statistics and described all continuous variables, e.g. number of fibers, fiber diameter, number of thick and thin fibers, fiber density, number of pores and pore size, with median and interquartile range (IQR, 25th to 75th percentile). Since the sample size was too small to detect statistically significant differences, we did not performed any statistical analysis. Medians and IQRs were calculated using SPSS version 24 (IBM, Armonk, NY, USA).

RESULTS

The 10 included women with major postpartum hemorrhage had a median age of 31 years [25 – 33 years] at time of hemostatic evaluation. Median blood loss was 3000 mL [2500 – 4000 mL]. Two women with major postpartum hemorrhage had low von Willebrand Factor (VWF) levels (VWF activity levels between 0.30-0.50 U/ml); no other hemostatic abnormalities were identified. The 5 women with an uncomplicated delivery had a median age of 43 years [36 – 44 years] at time of hemostatic evaluation. No hemostatic abnormalities were identified. Fibrinogen (2.7 g/L [2.4 – 3.2 g/L] versus 3.1 g/L [2.5 – 3.3 g/L], p=0.42) and FXIII-activity levels (1.18 U/ml [0.85 –1.45 U/ml] versus 1.17 U/ml [1.00 – 1.47 U/ml], p=0.74) were similar in patients and controls. No significant differences were found in other hemostatic variables measured.

Table 1. Demographic data of study population.

	Women with major PPH (n=10)	Women without PPH (n=5)	p
Age, y	31 (25 – 33)	43 (36 – 44)	0.01
N of pregnancies	3 (2 – 4)	2 (1 – 3)	0.61
N of miscarriages	1 (0 – 2)	0 (0 – 1)	0.24
Blood loss, mL	3000 (2000 – 7000)	< 500	n.a.
Blood type O, %	33%	50%	0.57
Abnormal bleeding score, %	60%	0%	0.03

Data are depicted as median (range), or %, as appropriate. A bleeding score was calculated using the International Sociatey of Thrombosis and Haemostasis – Bleeding Assessment Tool (ISTH-BAT), with a cut-off score of ≥ 6 or women. [20,21]

Table 2. Hemostatic variables in women with and without major postpartum hemorrhage (blood loss ≥ 2000 mL).

	Women with major PPH (n=10)	Women without PPH (n=5)	p
Hemoglobin, mmol/L	7.8 (7.5 – 8.2)	8.2 (7.1 – 8.7)	0.85
Plateletcount, 10^9/L	299 (229 – 364)	253 (209 – 332)	0.33
PFA – Col/EPI, sec	143 (127 – 179)	157 (113 – 184)	0.90
PT, sec	11.4 (11.2 – 12.2)	12.1 (12.0 – 13.3)	0.05
aPTT, sec	24 (23 – 25)	27 (25 – 28)	0.06
Fibrinogen, g/L	2.7 (2.4 – 3.2)	3.1 (2.5 – 3.3)	0.42
VWF:Act, U/ml	0.85 (0.73 – 0.96)	1.20 (0.66 – 1.31)	0.43
FVIII:C, U/ml	1.16 (1.00 – 1.29)	1.31 (0.97 – 1.95)	0.67
FXIII:C, U/ml	1.18 (0.85 – 1.45)	1.17 (1.00 – 1.47)	0.74

Hemostatic variables were measured ≥ 3 months postpartum. Data are depicted as median (IQR). PPH: postpartum hemorrhage; PFA: platelet-function analyzer; PT: prothrombin time; aPTT: activated partial thromboplasmin time; VWF: Von Willebrand factor; FVIII:C: factor VIII activity; FXIII:C: factor XIII activity.

The median number of fibers was similar in the two groups, 651 [597 – 701] in patients and 643 [611 – 677] (p=0.95) in controls. Median fiber diameter was 0.130µm [0.127 – 0.136 µm] for patients and 0.123µm [0.117 – 0.138µm] (p=0.39) for controls. The median number of thick (> 0.133µm) and thin (≤ 0.133µm) fibers in patients and controls was respectively 271 [237 – 294] and 337 [294 – 364] for patients and 199 [176 – 264] (p=0.11) and 400 [293 – 449] (p=0.39) for controls (see figure 2). The mean fiber density (percentage of area covered with fibers), pore size and number of pores in patients and controls were, respectively 13.4% [10.9 – 17.3%] versus 15.2% [11.4 – 28.0%] (p=0.46), 10.63 μm^2 [4.04 – 24.60 μm^2) versus4.00 μm^2 [2.35 – 14.98 μm^2] (p=0.33), and 638 [463 – 836] versus 752 [605 – 1010] (p=0.62) (see figure 3). These results did not change when two patients using the oral contraceptive pill at time of blood collection were excluded from the analysis.

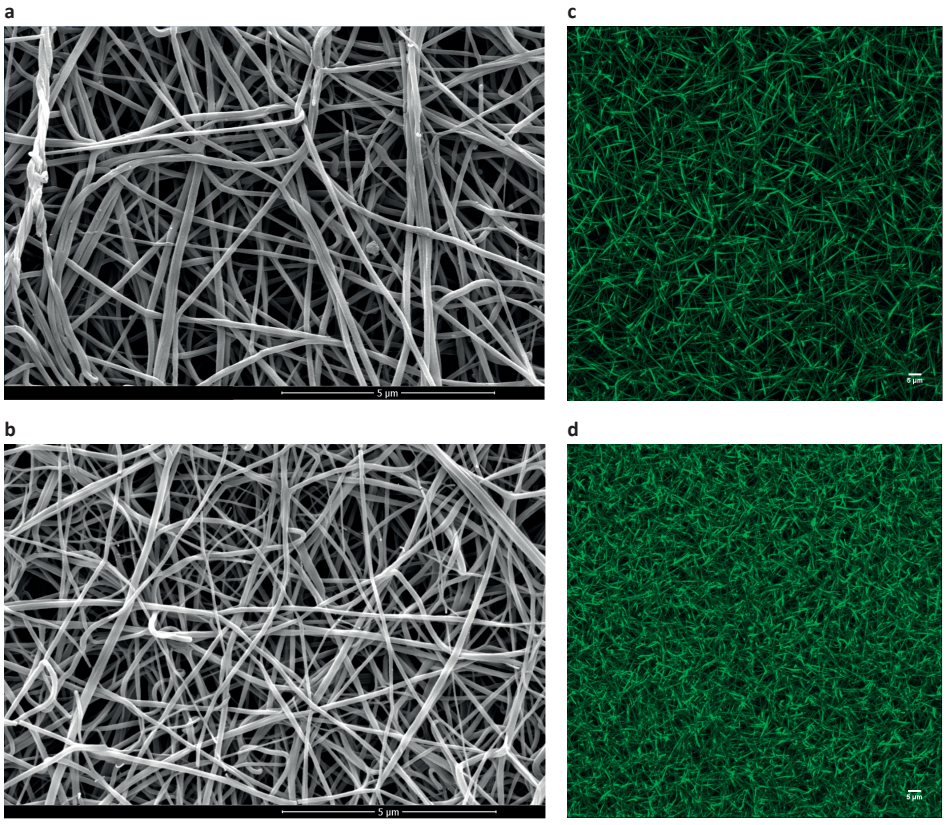

Figure 1a-d / table 3. Typical example of scanning electron microscopy and confocal laser scanning microscopy images and investigated clot specifics of a woman with and without experienced major postpartum hemorrhage.

	SEM – patient (fig 1a)	SEM – control (fig 1b)	LSCM – patient (fig 1c)	LSCM – control (fig 1d)
Fibrinogen, g/L	2.3	2.4	2.4	3.1
FXIII:C, U/ml	1.18	1.05	1.42	0.94
Mean density, %	-	-	12.29	22.02
Mean pore size, µm	-	-	16.73	2.43
Mean number of pores, n	-	-	522	910
Total number of fibers, n	570	705	-	-
Total number of think fibers, n	210	155	-	-
Total number of thin fibers, n	322	496	-	-
Mean fiber diameter, µm	0.126	0.114	-	-

Images a and c show a SEM (10.000x magnification) (a) and a confocal (c) micrograph (40x magnification) of a woman that experienced major PPH (≥2000ml blood loss within 24 hours after delivery). Images b and d shows a SEM (b) and a confocal (d) micrographs of a woman with an uncomplicated delivery. On both the SEM and confocal micrographs, the fibers of the women with PPH appear thicker, and the fibrin fiber network appears less dense with bigger pores, compared to the micrographs of the women without PPH.SEM: Scanning electron microscopy; LSCM: Laser scanning confocal microscopy; FXIII:C: factor XIII activity.

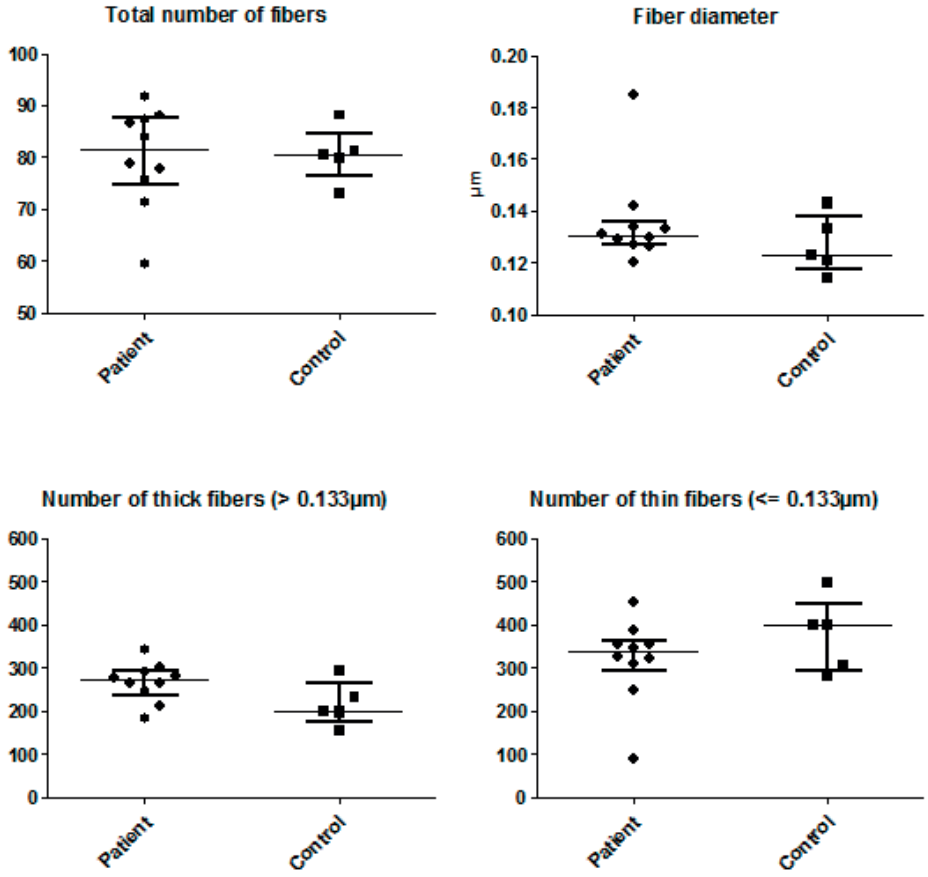

Figure 2. Total number of fibers (n), mean fiber diameter (µm), number of thick (>0.133µm) and thin (≤0.133µm) in women with and without PPH, measured using software package ImageJ (1.52b, Wayne Rasband, National Institutes of Health, USA).
The cut-off value of 0.133 µm is based on the mean fiber diameter of the total study group. Bars represent median, 25th and 75th percentiles.

DISCUSSION

To our knowledge, no studies have investigated fibrin clot structure in women with PPH before. It is important to realize that this study was designed as a pilot study, and therefore the number of participants and data is insufficient to detect any but the largest differences. Nevertheless, in this small group of women with PPH, clot characteristics such as the mean fiber diameter, number of thick and thin fibers, fiber density, and number and size of pores, from women that experienced major PPH did consistently trend in the direction that supports our hypothesis and were similar to findings described in several previous studies investigating clot structure in patients with coagulation factor deficiencies. The fibrin clots

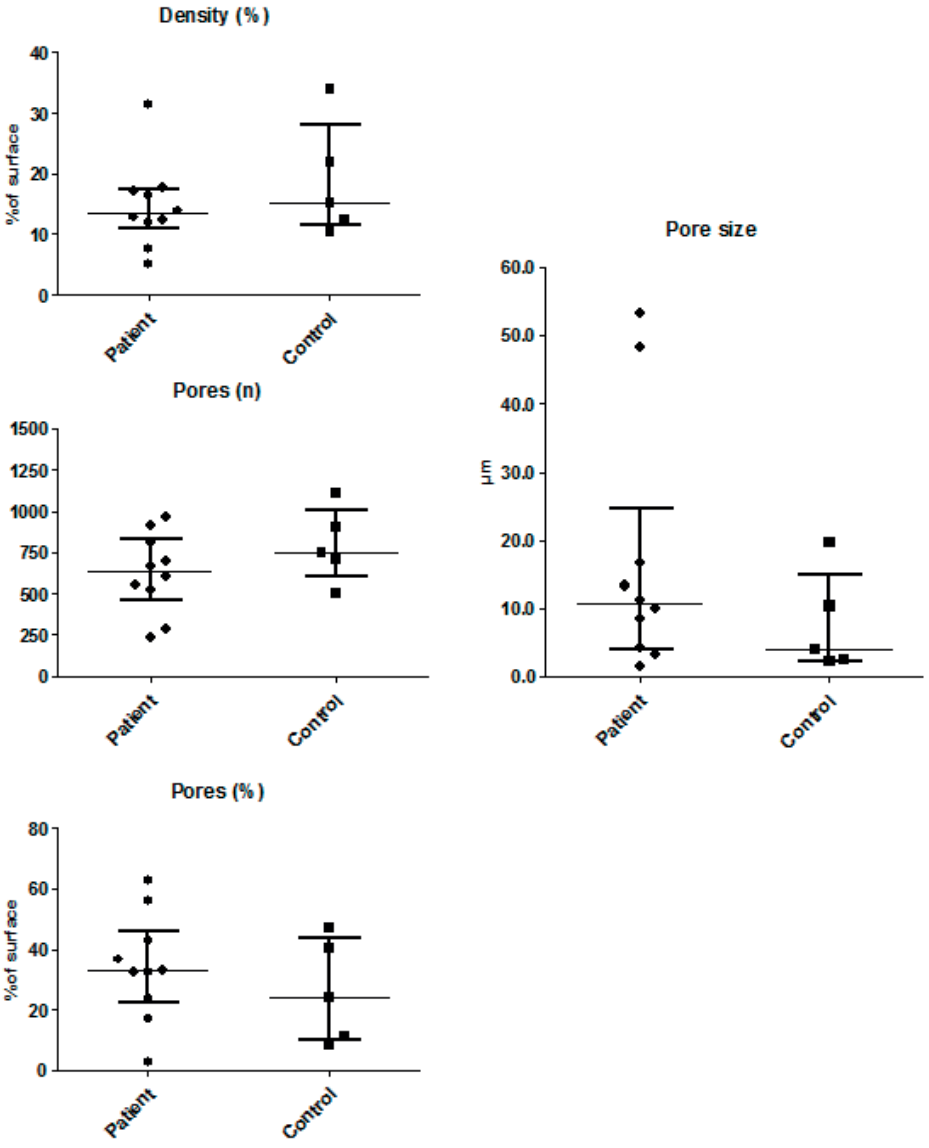

Figure 3. Fiber density of the clot (% of area of the clot covered with fibrin fibers), number of pores, percentage of pores (% of area of the clot covered with pores) and pore size (µm) in women with (patient) and without (control) postpartum hemorrhage.

Fiber density and pore size were obtained by an in-house designed macro (ImageJ 1.52b), and quantified by summing individual sections to create Z-projections and thresholding to visualize fibers and / or pores and minimize noise. The area covered by pixels corresponding to the set threshold cut-off was determined. Bars represent median, 25th and 75th percentiles.

acquired from our patients tended to have a reduced density, thicker fibers, and a larger pore size, compared to women with an uncomplicated delivery (see figure 1, 2 and 3). These observations suggest that clots of women with PPH possibly exhibit an abnormal structure, having clots with an increased susceptibility to fibrinolysis and to rupture from being weaker, caused by thicker fibers, reduced fiber density and larger pore size, causing severe blood loss after delivery.

It is known that fibrinogen rises with age. Although there was a significant difference in median age between the patient and the control group, fibrinogen-levels did not significantly differ between patients and controls. Therefore, we do not think this higher age in the control group affected our results.

Limited studies have been conducted investigating clot structure in bleeding disorders, in contrast to thrombotic disorders. The few studies that did investigate clot structure in bleeding disorders all show that different factor deficiencies cause clots with a reduced fiber density and relatively thick fibers. This was investigated in patients with hemophilia A (HA), in whom thicker fibrin fibers were found. After addition of FVIII or recombinant FVIIa, an increased fiber density, thinner fibers and more highly branched fibers resulting in smaller pore size, were found in hemophiliacs, compared to controls[12,13,17]. Also, clots from hemophiliacs have been shown to have a decreased clot stiffness, which can cause weaker clots more likely to rupture[17]. It is also shown that clots formed without FIX (Hemophilia B (HB) model) are composed of thicker fibrin fibers than those formed in the presence of FIX[14]. Factor-XI (FXI) deficient patients with an increased bleeding tendency have a reduced fibrin network density, compared to controls and nonbleeders[15]. Finally, clots in the presence of FXIII form significantly thinner fibers and have a higher density of fibers compared to those without FXIII[16].

With regard to FXIII cross-linking it is known that several identified polymorphisms, for example the relatively common Val34Leu polymorphism, can affect the function of FXIII, among others by increasing the rate of FXIII activation by thrombin and by altering the molecular structure of the cross-linked fibrin network. This, causing a denser fiber network, thinner fibers and altered permeation characteristics.[22-24] Although again mainly investigated in venous and arterial thrombotic disorders, the presence of such a polymorphism might also explain the difference in clot structure in patients with bleeding symptoms. This needs further investigation in selected populations, e.g. patients experiencing bleeding symptoms, without any laboratory abnormalities explaining these symptoms.

Currently, scanning electron microscopy and confocal laser scanning microscopy are not part of routine laboratory diagnostics in hemorrhagic disorders. Scanning electron microscopy is however the golden standard method to study fibrin clot structure. Measuring individual coagulation factors is often sufficient in patients with a severe bleeding tendency in order to diagnose bleeding disorders such as hemophilia. However, variation is observed in fibrin fiber diameter, fiber branching and the ability to form an interconnected meshwork

and different levels of FVIII that are required to achieve specific changes in clot stiffness in Hemophilia A patients, in response to FVIII replacement therapy[17]. This variation, which is also found in FXI deficient patients[14], as some patients bleed and some do not, can reflect differences in plasma coagulation factors, cellular elements including fibrinogen, and genetic polymorphisms that affect fibrin polymerization characteristics, and ultimately clot structure and viscoelastic properties. Therefore, investigation of fibrin clot structure with different imaging techniques and investigation of viscoelastic properties might predict the ability of clots to stop bleeding. In addition, there is a large group of patients with a mild bleeding tendency in whom no laboratory abnormalities can be identified. In this group of patients with Bleeding of Unknown Cause, which affects approximately 50% of patients with a mild bleeding tendency[25], it is not possible to diagnose a bleeding disorder. Postpartum hemorrhage can be a first presenting symptom of such a (mild) bleeding disorder. An inadequate interplay between several coagulation factors can cause a clot structure with specific characteristics making the clot less mechanically stable and less resistant to fibrinolysis. Therefore, techniques such as scanning electron microscopy or confocal laser scanning microscopy could be of additive value in patients with a (unexplained) bleeding tendency, in order to investigate global hemostasis and give direction for an adequate treatment regimen.

CONCLUSION

Fibrin clots acquired from women with major PPH tend to have a reduced density, thicker fibers, and a larger pore size, compared to clots from women with an uncomplicated delivery. These observations could suggest that the clots of women with major PPH possibly exhibit an abnormal structure, hereby having clots with an increased susceptibility to fibrinolysis, contributing to severe blood loss after delivery. However, this study was designed as a pilot study, and therefore the number of participants and data was insufficient to detect any but the largest differences. Further research, in order to confirm our findings, is recommended.

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CHAPTER 7

Thrombophilia: women-specific reference ranges can prevent misdiagnosis in women

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ABSTRACT

Background: Thrombophilia is a state where abnormalities of the haemostatic system predispose to thrombosis. Some coagulation factors are generally lower in women than in men. Therefore, the use of routine reference ranges (RRR), based on male or mixed-sex groups, may be misleading in the diagnosis of thrombophilia in women. We hypothesize that this affects the analysis of thrombophilia after pregnancy complications. Therefore the aim of our study was to investigate the effect of women-specific reference ranges (WRR) in the interpretation of haemostatic variables in postpartum women.

Methods: Coagulant andanticoagulant variables were measured three months postpartum in 61 healthy women with an uncomplicated pregnancy and in 197 women who experienced preeclampsia (PE). In 55 of the healthy women these variableswere also measured at least 6 months after an uncomplicated pregnancy and used to calculate WRR.

Results: There are no values outside of the reference ranges in anticoagulant factors and / or lupus anticoagulant – tests in 48% versus 89% of women, when compared to RRR and WRR, respectively ($p < 0.05$). When using RRR 26% of the women who suffered preeclampsia showed no abnormalities in anticoagulant factors and / or lupus anticoagulant – tests versus 67% when WRR were used ($p < 0.05$).

Conclusion: When using women-specific reference ranges less abnormalities are seen in healthy women as well as in women with a history of PE, which may prevent misdiagnosis of thrombophilia.

INTRODUCTION

Thrombophilia is a state where inherited or acquired abnormalities of the haemostatic system are present that predispose to thrombosis[1]. Inherited thrombophilias result from deficiencies of anticoagulant factors, and include antithrombin(AT), protein C (PC) and protein S (PS) deficiencies [2]. Acquired thrombophilia is often caused by the antiphospholipid syndrome, a diagnosis that is defined by a combination of clinical criteria and laboratory criteria. Clinical criteria are the presence of vascular thrombosis or pregnancy complications. Laboratory criteria are the presence of lupus anticoagulant (LAC) and / or anticardiolipin (aCL) and / or beta2-glycoprotein (β2GP) antibodies of either the IgG or IgM isotype (or both) on 2 or more occasions at least 12 weeks apart [3].

During a normal pregnancy major changes in haemostasis are seen. Briefly, pregnancy is associated with an increase in concentrations of most clotting factors, a decrease in concentrations of some of the natural anticoagulants and impaired fibrinolytic activity, which together induce a thrombophilic state, especially in the last trimester[4,5]. These changes in haemostasis, resulting from hormonal changes, protect women from fatal haemorrhage during delivery. The pathogenesis of several pregnancy complications is related with thrombosis, such as preeclampsia (PE)[6,7]. PE is characterized by a maternal hypercoagulable state with intravascular coagulation, microthrombosis in several organs and impairment of the uteroplacental circulation [8]. In 40 to 72% of preeclamptic women the presence of at least one thrombophilic factor after delivery has been reported[9-11].

To determine whether women who experienced a thrombotic event during pregnancy have a congenital or acquired haemostatic abnormality, analysis of haemostatic factors is usually performed after delivery[12] when the haemostatic abnormalities related to pregnancy are expected to be normalized[13-17]. It has been previously demonstrated, however, that some anticoagulants are lower in women than in men [16,18]. Also, immunoglobulin levels are higher in women than in men, and antibody production in response to primary and secondary antigen stimulations seems to be more pronounced in women[19]. Therefore, sex-specific reference ranges are recommended[16,20,21]. For example, in women compared to men, lower PS levels are reported and it has been shown that there is a higher frequency for positivity of IgM aCL antibodies with regard to the antiphospholipid syndrome[19]. Therefore, diagnosing thrombophilia in young postpartum women remains a challenge and the use of universal reference ranges may be misleading in these women[15,16,21]. We hypothesize that this affects the analysis of thrombophilia after pregnancy complications. Therefore the aim of our study was to investigate the effect of women-specific reference ranges (WRR) in the interpretation of haemostatic variables in postpartum women.

MATERIAL AND METHODS

Participants

In order to obtain women-specific reference ranges (WRR) we recruited healthy women from the Obstetric Department and the Birth Centre for low risk pregnancies of the Erasmus University Medical Center in Rotterdam, three months after an uncomplicated pregnancy (i.e. no preeclampsia (PE), HELLP syndrome or intrauterine growth retardation), from January 2009 to February 2011. The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center (Rotterdam, The Netherlands, MEC-2009-002) and the women were included after giving written informed consent in accordance with the Declaration of Helsinki.

PE-patients from the Obstetric Department of the Erasmus Medical Center Rotterdam are routinely investigated for thrombophilia three months postpartum. PE-patients visiting the department in the same time period (January 2009 to February 2011) and examined within the same postpartum time interval as the women with an uncomplicated pregnancy were included in this study. These women were retrospectively evaluated.

Blood collection

Blood was drawn in tubes containing 0.106 M citrate (1 part to 9 parts of blood) as anticoagulant and serum-tubes (BD Biosciences). Citrated samples were centrifuged two times, at 2000g for 10 minutes at 4°followed by 14000g for 10 minutes at 4° C and the plasma and serum were stored at -80°C until analysis. Testing for thrombophilia is performed 3 months after delivery, and therefore we collected blood samples from the healthy women as well as PE-women three months postpartum. In the healthy women a second sample was taken at least 6 months after delivery, since haemostatic markers are no longer influenced by the pregnancy [2,14,15,17]. At time of blood sampling, women provided information about the use of oral contraceptives (OC) and medication using a questionnaire.

Assays

Prothrombin time (PT; Thromborel S), Protein C Activity (PC), Activated Partial Thromboplastin Time Lupus, (aPTTL) and Lupus anticoagulans with diluted Russell's viper venom time (La-dRVVT) (Siemens Healthcare Products, Breda,Nederland); Antithrombin (AT-III; Chromogenix, ILC, Lexington, MA, USA), Protein S Activity (PS; Roche Diagnostica), and Activated Protein C resistance (APC-resistance; Chromogenix) were performed on Sysmex CA-1500 (Siemens HealthCare Diagnostics, Breda, Nederland). In-house ELISA assays were performed for the detection of anticardiolipin (ACL)-IgG and IgM and for beta 2-Glycoprotein I (β-2GP1)-IgG and –IgM antibodies.

DNA Analysis

Genomic DNA was extracted from blood leucocytes according to a standard procedure. Carriership of the gene variants FV R506Q (Factor V Leiden; FVL) and Prothrombin G20210A were determined by DNA amplification followed by restriction analysis as previously described[22,23].

Reference ranges

Routine reference ranges(RRR) used in our center are based on 40 healthy male blood donors with the lower limit mean + 2SD and the upper limit mean + 2SD or ± 3SD for lupus tests. WRR were calculated with the Reference Value Advisor Software (v2.1) which closely follows the CLSI guideline[24,25]. APC-resistance WRR was calculated based on women that were proven not to carry the FVL mutation.

Statistics

Continuous data were analysed with the Student's t-test (in case of a normal distribution) or a Mann-Whitney U test (in case of a skewed distribution). Categorical data were analysed using McNemar test for paired data and Pearson Chi Square test for unpaired data. All tests are two-tailed and groups were considered statistically significant if $p<0.05$. Analyses were carried out using IBM SPSS Statistics Data Editor, version 21.

RESULTS

Sixty-one women with an uncomplicated pregnancy were included. In all 61 women blood was collected 3 months postpartum (range 83-128 days). The median age at time of delivery was 32.2 years (range 18.2 – 39.1). In 55 of the 61 (90%) women a second blood sample was collected at least 6 months postpartum (range 181 – 280 days). The women-specific reference ranges (WRR) were determined based on this last measurement (table 1). Three and six months after delivery, 18% and 27% of the women used oral contraceptives, respectively.

Table 1. Characteristics of women included in the study

	Group H		
	Group H3	Group H6	Group P
N	61	55	197
Age at delivery (years)	32.3 (18.2 – 39.1)	N.A.	31.3 (19.2 – 44.7)
Days after delivery	97 (83 – 128)	214 (181 – 280)	91 (83 – 142)
Use of oral contraceptives	11 (19%)	15 (27%)	-
Use of over-the-counter medication	7 (11%)	-	-

Data are presented as median (range) or numbers (%), as appropriate. H3: healthy women three months postpartum; H6: healthy women six months postpartum; P: patient group, women with preeclampsia.

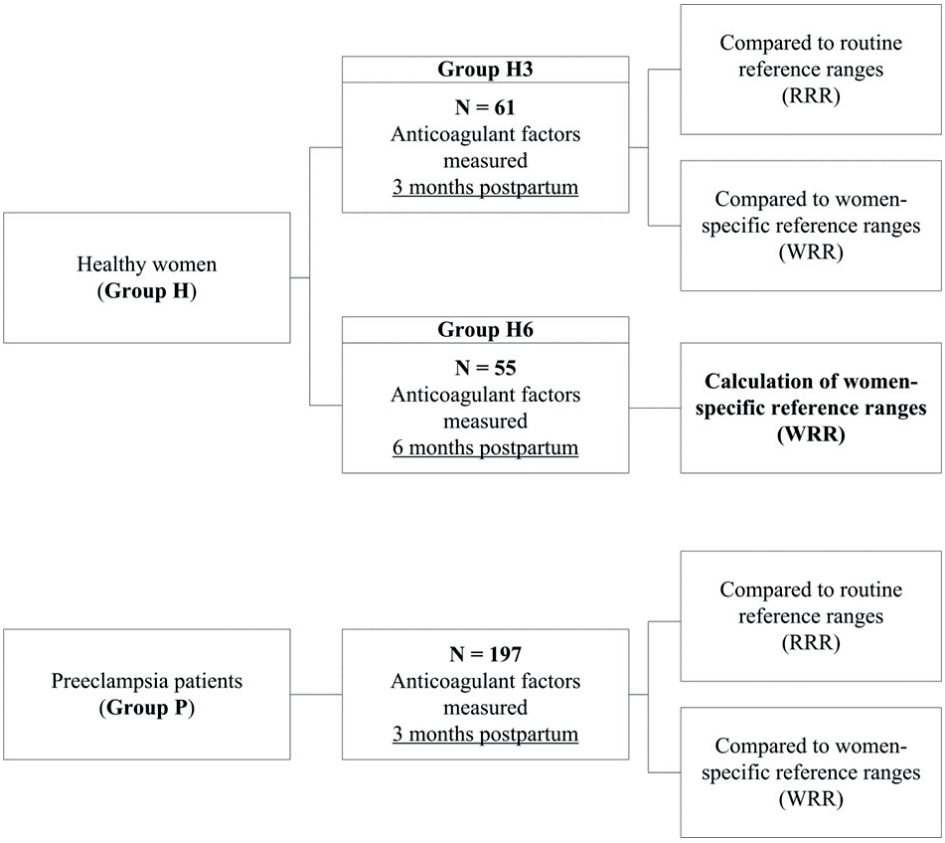

Figure 1. Flowchart of inclusion of healthy women and preeclampsia patients
Inclusion of healthy women (group H) and preeclampsia patients (group P). In group H anticoagulant factors were measured three (group H3) and six months postpartum (group H6). Based on the measurements six months postpartum women-specific reference ranges were calculated.

Women-specific reference ranges

Two women with an uncomplicated pregnancy carried the gene variant FV R506Q (Factor V Leiden; FVL). A total of 22 women were proven not to carry FVL. No carriers of the Prothrombin G20210A gene variant were seen in this cohort (tested in 28 women). The women-specific cut-off value of APC resistance ratio was 0.58, lower than compared to the cut-off value of 0.80 used as RRR.

The WRR for AT (0.69 – 1.37 U/ml), was somewhat wider than the RRR (0.80 – 1.20 U/ml). For PC activity the lower limit of the WRR was higher (0.75 – 1.43 U/ml) than the lower limit of the RRR (0.70 – 1.30 U/ml). In contrast, for PS activity the lower limit of the WRR (0.57 – 1.20 U/ml) was lower that the lower limit of the RRR (0.70 – 1.30 U/ml).

The upper limits of the WRR for APTT-Lupus (42 seconds versus RRR 39 seconds), DRVVT Ratio (<1.26 versus RRR: <1.20), aCL IgM antibody (50 U/ml versus RRR: <23 U/ml), and

Table 2. Coagulation factors in women with an uncomplicated pregnancy – routine reference ranges (RRR) and women-specific reference ranges (WRR)

	Routine Reference Ranges (RRR)	Women-specific Reference Ranges (WRR)[a]
Antithrombin (U/ml)	0.80 – 1.20	0.69 – 1.37
Protein C activity (E/ml)	0.70 – 1.30	0.75 – 1.43
Protein S activity (E/ml)	0.70 – 1.30	0.57 – 1.20
APC-resistance ratio	> 0.8	0.60 – 1.14
APTT-Lupus (sec)	≤ 39 sec	≤ 42 sec
DRVVT Ratio	< 1.20	<1.26
ACL-IgG (U/ml)	< 21	<13
ACL-IgM (U/ml)	< 23	<50
β-2GP1-IgG (U/ml)	< 30	<45
β-2GP1-IgM (U/ml)	< 15	<40

Data are presented as mean ± SD. Abbreviations: APC: activated protein C; APTT: activated partial thromboplastin time; DRVVT: diluted Russell's viper venom time; ACL: anticardiolipin; β-2GP1: beta 2-glycoprotein. [a]Women-specific reference ranges were calculated with the Reference Value Advisor software using coagulation factors measured in healthy women 6 months after an uncomplicated pregnancy.

β2GP-1IgG and IgM antibody (45 U/ml and 40 U/ml respectively versus RRR: < 30 U/ml and < 15 U/ml respectively) were all higher than the routine reference cut-off values. In contrast, the aCL IgG antibody upper limit of the WRR was lower (13 U/ml) compared to the RRR (21 U/ml)(tabel 2).

When analysing haemostatic variables three months postpartum in women with an uncomplicated pregnancy, the number of values outside of the reference ranges depends on the specific reference ranges used. There are no values outside of the reference ranges in anticoagulant factors and / or lupus anticoagulant – tests in 48% versus 89% of women, when using RRR and WRR, respectively ($p < 0.05$)(table 4).

Oral contraceptive use and women-specific reference ranges

In our study population the women that used oral contraceptives had a significantly decreased antithrombin level six months after pregnancy. We did not find a significant difference in the other anticoagulant factors between women using oral contraceptives and women not using oral contraceptives. The use of oral contraceptives however, did had an effect on the WRR for all thrombophilia factors (table 5).

Preeclampsia and thrombophilia

One hundred and ninety seven women who suffered from PE during their last pregnancy were included. Blood was collected 3 months postpartum (range 83 – 142 days) as part of routine care. The median age of the PE women at time of delivery was 31.3 years (19.2 – 44.7) (table 1). When using RRR, 26% of the women who suffered preeclampsia showed

no abnormalities in anticoagulant factors and / or lupus anticoagulant – tests, versus up to 67% when WRR were used (p < 0.05) (table 4). The most pronounced abnormalities were found regarding PS activity (32 versus 4 abnormal values), APC-resistance ratio (88 versus 15 abnormal values) and DRVVT ratio (54 versus 30 abnormal values) (table3b).

Table 3. Total of abnormal laboratory results based on routine reference ranges (RRR) and women-specific reference ranges (WRR)

	Group H3			Group P		
	RRR	WRR	p	RRR	WRR	P
Anticoagulant factors						
No deficiencies	29 (48%)	57 (93%)	< 0.05	90 (46%)[a]	170 (87%)	< 0.05
Lupus anticoagulant tests						
All negative	52 (85%)	56 (92%)	n.s.	113 (57%)	153 (78%)	< 0.05
Total						
No abnormalities	29 (48%)	53 (87%)	< 0.05	51 (26%)[b]	129(66%)	< 0.05

Data are presented as numbers (%).

Table 4. Number of abnormal laboratory results in healthy women (group H3) and preeclampsia patients (group P) 3 months postpartum based on routine reference ranges (RRR) and women-specific reference ranges (WRR)

	Group H3				Group P			
	RRR		WRR		RRR		WRR	
Antithrombin (U/ml)	1	(2%)	0	(0%)	3	(2%)	1	(0,5%)
Protein C activity (E/ml)	1	(2%)	2	(3%)	5	(3%)	8	(4%)
Protein S activity (E/ml)	7	(11%)	0	(0%)	32	(16%)	4	(2%)
APC-resistance ratio	26	(43%)	2	(2%)	88	(45%)	16	(8%)
APTT-Lupus (sec)	1	(2%)	0	(0%)	2	(1%)	1	(0,5%)
DRVVT Ratio	5	(8%)	1	(2%)	54	(27%)	30	(15%)
ACL-IgG (U/ml)	0	(0%)	3	(5%)	8	(4%)	9	(5%)
ACL-IgM (U/ml)	1	(2%)	1	(2%)	0	(0%)	0	(0%)
β-2GP1-IgG (U/ml)	0	(0%)	0	(0%)	22	(11%)	10	(5%)
β-2GP1-IgM (U/ml)	2	(3%)	0	(0%)	29	(15%)	3	(2%)

Data are presented as numbers (%). Abbreviations: APC: activated protein C; APTT: activated partial thromboplastin time; DRVVT: diluted Russell's viper venom time; ACL: anticardiolipin; β-2GP1: beta 2-glycoprotein.

DISCUSSION

The main result of our study is that over 40% of women would be falsely classified as having thrombophilia using routine reference ranges. Significantly more normal results are seen in women after an uncomplicated pregnancy and preeclampsia when using women-specific

reference ranges (89% and 67% respectively) compared to the use of routine haemostatic reference ranges (48% versus 26% respectively), based on a healthy male or mixed-population blood donors. This finding indicates that women-specific reference ranges should be used for the interpretation of haemostatic variables in this group as false classification can have major consequence for future pregnancies, and other situations with an increased thrombosis risk, throughout life.

Our finding that a difference in reference ranges for women compared to men is seen, is in line with literature. Previously reported data consistently show that gender has an influence on coagulation factors[16,18,21,26,27]. With this present study we concur with the suggestion of Lowe et al.[21] that sex specific reference values at least should be considered in the diagnosis of congenital thrombophilias, as a significant difference is seen in our study group. Furthermore, we also found that the total number of abnormalities in the lupus anticoagulant – tests and positivity of antiphospholipids in our study group differ when using women-specific reference values compared to the routine reference group. This is not yet described in literature and can have an impact on the diagnosis of lupus or antiphospholipid syndrome.

Our study has several limitations and strengths. In order to determine the women specific reference ranges, we included 55 healthy women with an uncomplicated pregnancy. Currently, guidelines recommend samples of 120 individuals for interval determination. Very often however, it is not possible to obtain the suggested number of 120 individuals of a specific group to define the reference ranges[28]. Therefore, the revised CLSI guideline has introduced determination of reference ranges from smaller reference samples based on a robust method, preferably after transformation of the data to a distribution that is closer to Gaussian or normal[24,25]. We chose to use the Reference Value Advisor software for determining women-specific reference ranges, which is guided by the IFCC-CLSI recommendations[25] and permits evaluation and transformation of data distributions and computation of reference ranges with the corresponding confidence intervals.

In our study population the use of oral contraceptive resulted in a significant decrease of antithrombin (AT) six months after pregnancy, while other factors were not different. This is consistent with previous papers that reported that the use of oral contraceptives has an influence on coagulation factors[16,18,21,26,27]. An hormonal influence on the decrease of protein S(PS) plasma levels was already described in 1987 by Boerger et al[20]. Also, in our study the use of oral contraceptives also had an effect on the women specific reference ranges for all thrombophilia factors. That we did not find significant differences in anticoagulant factors, other than antithrombin, between users and non-users of oral contraceptives could be explained by the relatively small number of women on oral contraceptives included in our study.

However, since the contraceptive pill is used in a large group of young women (37% of women between 18 and 45 years of age in the Netherlands in 2013[29]), we did include women

using oral contraception in the women-specific reference ranges. As the diagnostic process of congenital thrombophilia is challenging and is not necessarily carried out in the routine laboratory, a physician other than the treating physician is often consulted. Information on the use of oral contraception may not always be known to the consulted physician. The differences in AT, protein C (PC) or PS plasma levels associated with gender and hormonal status however, might be of clinical relevance in the interpretation of low borderline results. In fact, when levels of anticoagulant factors fall in this grey area, these patients are quite often diagnosed as carriers of an inherited deficiency, while they can simply be part of a group with slightly lower (but still normal) levels depending on their gender or hormonal status. Nevertheless, whether or not these low borderline levels represent a true risk factor for thrombosis is another important question, yet to be established[18,30]. Furthermore, as the diagnostic process of congenital thrombophilia is challenging and most of the assays require specialized materials or a complicated methodology they are not necessarily carried out in the routine laboratory.

CONCLUSION

The use of women-specific reference ranges should allow a more accurate definition of true congenital thrombophilia and prevent a misclassification of thrombophilia which can have aggravating clinical consequences.

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CHAPTER 8

Anticoagulation control in premenopausal women with a mechanical heart valve using vitamin K antagonists: room for improvement

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ABSTRACT

Objective: High quality of anticoagulation control with vitamin K antagonists (VKAs) is essential for safe and effective treatment in patients with a mechanical heart valve (MHV). In premenopausal women, variability in levels of coagulation factors due to the menstrual cycle may be associated with a less optimal anticoagulation control. The aim of this study was to investigate anticoagulation control in young women with MHV on VKAs.

Methods: In this retrospective cohort study, patients with MHV treated with VKAs, monitored by a Dutch anticoagulation clinic between 2005 and 2015 were included. Percentage of time in therapeutic range (TTR), cross-sectional proportion (CSP) and incidence rates of major clinical events were compared between younger (16-45y) and older (≥ 55y) women and age-matched men.

Results: In total 1177 MHV patients were eligible for inclusion, of whom 41% were female. Thirteen percent of patients was younger than 45 years during treatment with VKA. Younger women had a significantly lower TTR and CSP (64.5% and 51.9%) compared to older women (71.5% and 71.0%; both $p<0.05$) and significantly lower TTR compared to young men (70.1%, $p<0.05$). Hazard ratios for clinical events were not clearly different.

Conclusion: Younger women had a lower quality of anticoagulation control, compared to older women and age-matched men. As these patients are at significant risk of complications, optimization of VKA therapy in young women is of great importance.

INTRODUCTION

Mechanical heart valves (MHVs) are more durable than bio-protheses, but also more thrombogenic leading to a substantially higher risk of thrombosis and systemic embolism[1-3]. For this reason, long-term management with oral anticoagulant therapy with vitamin K antagonists (VKAs) is recommended[3]. Due to this substantially higher risk of thrombosis and systemic embolism [4], both European and American guidelines[3,5,6] recommend high intensity anticoagulation (INR target of 3.0) in these patients. The use of DOACs in this specific patient group is contraindicated[3,5], as dabigatran was shown to be inferior to warfarin in patients with a mechanical heart valve, both in terms of efficacy (e.g. ischemic stroke risk) and safety (e.g. bleeding risk)[7].

Anticoagulant treatment is associated with an increased bleeding risk, especially at supra-therapeutic INR levels, and with increased thrombotic risk at subtherapeutic INR levels[4,8-10]. The quality of VKA treatment is expressed as the percent of time in therapeutic INR range (TTR)[11], which is also correlated to thrombotic and bleeding events[6]. Therefore, stability of INR and high quality of anticoagulation control are essential for safe and effective treatment. Predictors of poor anticoagulation control have been studied in the past. Among others, higher intensity of therapeutic range and long intervals between measurements are identified as risk factors for poor anticoagulation control[12,13]. It has also been shown that young age and female sex are predictors of lower quality of anticoagulation control and higher risk of complications during VKA therapy[14-16]. The most important determinant of INR stability during treatment with VKAs is the level of coagulation factor VII (FVII), which is strongly related to the half-life of the used VKA (acenocoumarol versus warfarin)[17,18]. Several studies have reported hormone dependent fluctuations of coagulation factor levels during the menstrual cycle, including FVII[19,20].

The difference in anticoagulation control between pre- and postmenopausal women, and the impact of cyclic fluctuations of coagulation factors (including FVII) on INR stability and quality of anticoagulation control in women treated with VKAs, has not yet been studied. We hypothesize that lower quality of anticoagulation control in women and younger patients is caused by fluctuation of coagulation factors during the menstrual cycle. In order to explore this hypothesis, we assessed anticoagulation control in young women with a MHV on VKA treatment, and compared this group with older women and age-matched men.

MATERIAL AND METHODS

Patients

In this retrospective cohort study, patients receiving high-intensity (therapeutic INR range, 2.5 – 4.0) treatment with VKA for a MHV, monitored by the thrombosis service of Star-shl

(Rotterdam, The Netherlands) and started VKA therapy between 2005 and 2015, were eligible for inclusion. The included patients were divided into two groups, a group of patients aged 16 – 45 years and a group of patients aged 55 years and older. Because of uncertain menopausal status, women between 46 and 54 years were excluded. Also, patients having a low treatment intensity (target INR between 2.5 and 3.5), and patients having less than 7 INR measurements during the follow-up period were excluded. This study was based on patients monitored by a thrombosis service, self-monitoring patients were not included. Of the included patients, all INR measurements and major clinical events (major bleeding, ischemic stroke, or death) between 2005 and 2015 were collected. All patients were followed until they ended treatment with VKA, died or reached the end of the observation period (31 December 2015). During the observation period, frequency of monitoring and VKA dosage was based on the patients INR results and adjusted if necessary according to the same acenocoumarol and phenprocoumon dosing schedules, according to the guidelines of the federation of Dutch Thrombosis Services[21].

Data collection

We retrieved data from patient records of the thrombosis service of Star-shl (Rotterdam, the Netherlands). INR results, changes in VKA dose and information obtained during patient visits were registered in the clinic's electronical medical database. Major clinical events (major bleeding, ischemic stroke, and all-cause mortality) were either registered during patients visits or reported directly or in retrospect to the clinic by the treating physicians. The outcome and severity of all adverse events were registered by specialized physicians as part of usual medical care of the clinic. These physicians were not involved in the current study. The medical board of the Star-shl approved the use of coded patient data and the study was conducted in accordance with the Helsinki declaration. The ethics committee of the Erasmus University Medical Center granted a waiver for informed consent due to the observational nature of the study. Based on the retrospective and observational nature of this study, this research was done without patient involvement.

Outcomes

Quality of anticoagulation control was defined as percentage of time in therapeutic range (TTR) and as cross-sectional proportion (CSP). TTR was calculated using the Rosendaal method[11]. If consecutive INR measurements were more than 56 days apart, the monitoring period was censored and not included in TTR analysis. To determine complications during the study period, we computed incidence rates of major bleeding, ischemic stroke, and all-cause mortality from moment of inclusion until the observation period ended (31 December 2015). All patients were followed until they ended treatment with VKA, died or reached the end of the observation period. Major bleeding was defined as any fatal or intra-articular or intracranial hemorrhage, or a bleeding that required hospitalization or blood transfusion. Secondary

outcomes were frequency of INR testing per patient-year, percentage of INR results within therapeutic range, and percentage of INR results followed by a significant dose-adjustment, defined as any dose adjustment of 10% or more[22]. Outcomes of younger premenopausal women (≤ 45 years) were compared with older postmenopausal women (aged ≥ 55 years) and age-matched men (aged between 16 and 45 years and aged ≥ 55 years).

Statistical analysis

We used descriptive statistics to summarize baseline characteristics of the study group. In case of a skewed distribution, data are presented as median and interquartile range (IQR), and compared by Mann-Whitney U test. In case of a normal distribution, data are presented as mean and standard deviation (SD), and compared using an independent sample *t*-test. Categorical data are presented as numbers with percentages and compared using a Pearson Chi-squared test. For clinical events, incidence rates and 95% confidence intervals (CIs) were calculated based on the Poisson distribution. In addition, Kaplan-Meier curves were constructed for each exposure group to compare cumulative incidence of major clinical events and hazard ratios and 95% confidence intervals were estimated by means of a Cox proportional hazard model. A p-value of < 0.05 was considered statistically significant. All analyses were performed with SPSS version 24.0 (IBM, Armonk, NY, USA).

Sensitivity analysis

We performed a sensitivity analysis to verify the validity of our findings. A sensitivity analysis was performed on patients with a MHV who were treated with VKA for at least six months, because it is known that TTR in the inception period (first six months of treatment with VKA) is significantly lower[23].

RESULTS

In total, 1790 patients were eligible for inclusion. After exclusion of patients aged 46 to 54 years, patients having a low treatment intensity (target INR between 2.5 and 3.5), and patients having less than 7 INR measurements during the follow-up period, a total 1177 patients were included in this study (see figure 1). For study group characteristics see table 1. Patients had a mean age of 64 years (SD 14.6), and 41% was female. Thirteen percent of patients was 45 years of age or younger. In total, 63 women aged 45 years or younger were included for analysis (see figure 1 and table 1). The 1177 patients had a total of 2802 patient-years of treatment with a VKA for a MHV, with 58% of patients being treated for more than six months (see table 1 and 2). Almost all patients used a short acting VKA (acenocoumarol, 93%) (see table 1). No significant difference were found in number of INR measurements per person year between men and women, in both age-categories (see table 2).

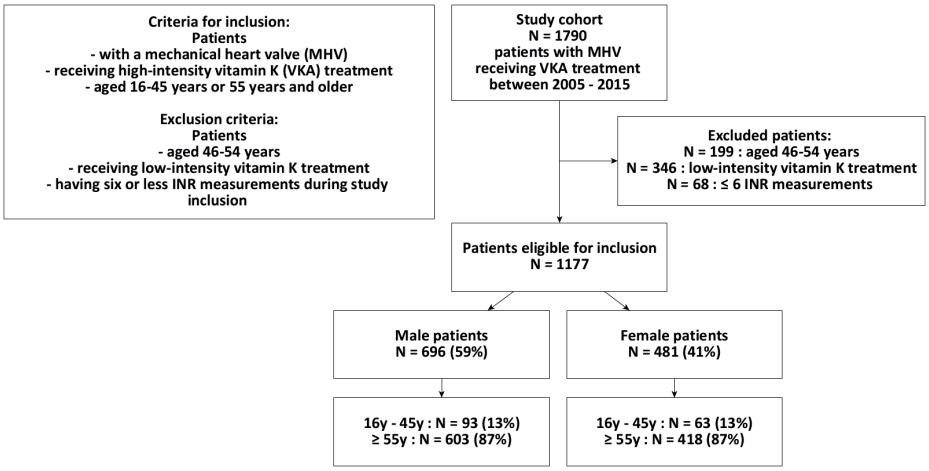

Figure 1. Flowchart of inclusion
Flowchart of study protocol and number of included patients, according to sex and age.

Table 1 Patient characteristics

N	**1177**
Mean age (SD), years	64.0 (14.6)
16 - 45 years of age, n (%)	156 (13%)
≥ 55 years of age, n (%)	1021 (87%)
Female	481
16 - 45 years of age, n (%)	63 (14%)
≥ 55 years of age, n (%)	418 (86%)
Type of VKA, n (%)	
Acenocoumarol	1099 (93%)
Phenprocoumon	77 (6.9%)
Warfarin	1 (0.1%)
Treatment duration, n (%)	
< 6 months	489 (42%)
Female, n (%)	198 (40%)
Female 16 - 45 years of age, n (%)	27 (14%)
≥ 6 months	688 (58%)
Female, n (%)	283 (41%)
Female 16 - 45 years of age, n (%)	36 (13%)

Abbreviations: SD = Standard deviation, VKA = Vitamin-K antagonist

Table 2 Number of INR measurements per person year, male versus female

	Male				Female				
	N	Person years	Nr of INR measurements, n	Nr of INR measurements per person year (95%-CI)	N	Person years	Nr of INR measurements, n	Nr of INR measurements per person year (95%-CI)	p
16-45y	93	163	3936	23.6 (23.4 – 23.9)	63	134	3984	26.3 (26.0 – 26.6)	n.s.
>=55y	603	1481	34924	24.2 (23.4 – 29.4)	418	1024	26451	29.7 (28.8 – 30.7)	n.s.
Total	696	1644	38860	23.6 (23.3 – 23.8)	481	1158	30435	25.8 (25.5 – 26.1)	n.s.

TTR and CSP analyses

For TTR, a significantly lower TTR was found in younger women (64.5%, IQR 56.5 – 74.2%) compared to older women (71.5%, IQR 61.7 – 79.7%, p=0.00) and age-matched men (70.1%, IQR 61.1 – 80.3%, p=0.02). Overall, median TTR was significantly lower in women (70.6%, IQR 59.9 – 79.3) than in men 73,7%, IQR 61.8 – 81.3; p=0.03). There was no difference in TTR between younger and older men nor between older men and older women (see table 3a). Overall, younger patients (men and women combined) had a significantly lower TTR (68.4%, IQR 58.2 – 78.7%) compared to older patients (73.3%, IQR 61.8% - 80.9%, p=0.005)(data not shown).

For cross-sectional proportion (CSP), younger women had a significantly lower CSP (51.9%, IQR 0.0 – 82.1%) compared to older women (71.0%, IQR 50.0 – 100%, p<0.01). No difference was found between younger women and age-matched men (66.7%, IQR 33.3 – 100%; p=0.07). In addition, there was no difference in CSP in males (68.8%, IQR 50.0 – 100%) compared to females (66.7%, IQR 42.9 - 100%, p=0.78), overall. Furthermore, no differences in CSP were found comparing younger men to older men and comparing older men and women (see table 3a).

Secondary outcomes

There were significant differences in the number of significant dose-adjustments (>10%) in all age groups when comparing women to men. Overall men had 5.3% (IQR 1.3 – 12.5%) of INRs followed by a significant dose-adjustment versus 7.1% (IQR 2.8 – 14.3%) in women (p<0.001), younger men had 4.9% (IQR 0.0 – 11.2%) of INRs followed by a significant dose-adjustment versus 9.1% (IQR 3.0 – 14.8%) in younger women (p=0.02); and older men 5.3% (IQR 1.3 – 12.9%) versus older women with 7.1% (IQR 2.8 – 14.3%)(p=0.02). No significant differences were found in dose-adjustments between young and older men, and between younger and older women (see table 4).

There was a significant difference between younger women (57.6%, IQR 48.5 – 64.3%) versus older women (61.5%, IQR 51.4 – 71.5% p=0.000) in the percentage of all INR results within therapeutic range. Also, there were significant differences in the percentage of all INR results within therapeutic range, with women in all age categories having a significantly

Table 3a Time in therapeutic range (TTR, %) and cross-sectional proportion (CSP, %), male versus female

	Male	Female		Male	Female	
	Median TTR, % [IQR]	Median TTR, % [IQR]	P*	CSP, % [IQR]	CSP, % [IQR]	P*
16-45y	70.1% [61.1 – 80.3%]	64.5% [56.5 – 74.2%]	*0.022*	66.7% [33.3 – 100%]	51.9% [0.0 – 82.1%]	*n.s.*
>=55y	74.0% [62.0 – 81.3%]	71.5% [61.7 – 79.7%]	*n.s.*	69.0% [50.0 – 100%]	71.0% [50.0 – 100%]	*n.s.*
p[a]	*n.s.*	*0.002*		*n.s.*	*0.010*	
Total	73.7% [61.8 – 81.3%]	70.6% [59.9 – 79.3%]	*0.034*	68.8% [50.0 – 100%]	66.7% [42.9 – 100%]	*n.s.*

*Mann-Whitney U. Abbreviations: TTR = Time in therapeutic range, CSP = Cross sectional proportion.

Table 3b Time in therapeutic range (TTR, %) and cross-sectional proportion (CSP, %), male versus female treated with VKA ≥ 6 months

	Male	Female		Male	Female	
	Median TTR, % [IQR]	Median TTR, % [IQR]	P*	CSP, % [IQR]	CSP, % [IQR]	P*
16-45y	72.2% [64.1 – 79.6%]	66.3% [57.7 – 72.5%]	*0.030*	69.1% [50.0 – 100%]	51.9% [30.8 – 71.4%]	*0.011*
>=55y	75.0% [66.6 – 81.1%]	74.0% [65.4 – 79.8%]	*n.s.*	66.7% [50.0 – 84.3%]	66.7% [50.0 – 85.7%]	*n.s.*
p[a]	*n.s.*	*0.001*		*n.s.*	*0.004*	
Total	75.0% [67.4 – 81.3%]	72.4% [64.3 – 79.1%]	*n.s.*	66.7% [50.0 – 85.7%]	66.7% [50.0 – 85.7%]	*n.s.*

*Mann-Whitney U. Abbreviations: TTR = Time in therapeutic range, CSP = Cross sectional proportion.

lower percentage of INR results in therapeutic range (overall male 63.6% (IQR 52.5 – 72.5%) versus female 60.4% (IQR 51.1 – 70.8%), p=0.02; younger men 64.0% (IQR 52.9 – 72.6%) versus younger women 57.6% (IQR 48.5 – 64.3%), p=0.02; and older men 63.6% (IQR 52.3 – 72.5%) versus older women 61.5% (IQR 51.4 – 71.5%), p<0.001) (see table 4).

Incidence rates and hazard ratios of major clinical events during follow-up are shown in table 4. The occurrence of clinical events was comparable for men and women (figure 2, all log-rank p values > 0.05). Significant differences were found in all-cause mortality between men and women and between younger and older patients (log-rank p value 0.004 for men versus women overall, log-rank p value of 0.027 for younger women versus older women). Hazard ratio for younger women as compared to younger (age-matched) men was 0.82 (95% CI 0.07 – 9.04) for major bleeding. Hazard ratio for older women as compared to older men were 1.11 (95% CI 0.67 – 1.85) for major bleeding, 0.77 (95% CI 0.26 – 2.30) for ischemic stroke, and 0.97 (95% CI 0.65 – 1.46) for all-cause mortality (see table 5).

Sensitivity analysis

Sensitivity analysis showed similar results as the main analyses, i.e. when treated more than six months with a VKA, also a significantly lower TTR in young women compared to older women and age-matched men was found. Same results were found for CSP, with significantly lower CSP in younger women than older women and age-matched men (see table 3b).

Table 4 Percentage of INR results followed by a significant dose-adjustment (> 10%) and percentage of INR results within therapeutic range (PP INR, %), male versus female

	Male	**Female**		**Male**	**Female**	
	Dose adjustments >10%, % [IQR]	Dose adjustments >10%, % [IQR]	P*	PP INR, % [IQR]	PP INR, % [IQR]	P*
16-45y	4.9% [0.0 – 11.2%]	9.1% [3.0 – 14.8%]	*0.020*	64.0% [52.9 – 72.6%]	57.6% [48.5 – 64.3%]	*0.015*
>=55y	5.3 [1.3 – 12.9%]	7.1% [2.8 – 14.3%]	*0.015*	63.6% [52.3 – 72.5%]	61.5% [51.4 – 71.5%]	*n.s.*
p[a]	*n.s.*	*n.s.*		*n.s.*	*0.013*	
Total	5.3% [1.3 – 12.5%]	7.1% [2.8 – 14.3%]	*0.002*	63.6% [52.5 – 72.5%]	60.4% [51.1 – 70.8%]	*0.024*

*Mann-Whitney U. Abbreviations: PP INR = Percentage of all INR results within therapeutic range.

Table 5 Hazard ratios of major clinical events during treatment period

			Patients	**Events**	**Person-years**	**Incidence rate per 1000py (95% CI)**	**Hazard ratio (95% CI)**
Mortality (all-cause)	Male	16-45	93	1	163	6.1 (0.3 – 30.3)	Reference
		≥55	603	57	1481	38.5 (29.4 – 49.5)	Reference
		Total	696	58	1644	35.3 (27.0 – 45.3)	Reference
	Female	16-45	63	0	134	-	-
		≥55	418	41	1024	40.0 (29.1 – 53.8)	0.97 (0.65 – 1.46)
		Total	481	41	1158	35.4 (25.7 – 47.6)	1.01 (0.67 – 1.52)
Major bleeding	Male	16-45	93	2	155	12.9 (2.2 – 42.6)	Reference
		≥55	603	35	1363	25.7 (18.2 – 35.3)	Reference
		Total	696	37	1518	24.4 (17.4 – 33.2)	Reference
	Female	16-45	63	1	134	7.5 (0.4 – 36.8)	0.82 (0.07 – 9.04)
		≥55	418	26	947	27.5 (18.3 – 39.7)	1.11 (0.67 – 1.85)
		Total	481	27	1081	25.0 (16.8 – 35.8)	1.06 (0.64 – 1.74)
Ischemic stroke	Male	16-45	93	0	163	-	Reference
		≥55	603	9	1410	6.4 (3.1 – 11.7)	Reference
		Total	696	9	1573	5.7 (2.8 – 10.5)	Reference
	Female	16-45	63	0	134	-	-
		≥55	418	5	1016	4.9 (1.8 – 10.9)	0.77 (0.26 – 2.30)
		Total	481	5	1050	4.8 (1.7 – 10.6)	0.75 (0.25 – 2.25)

Abbreviations: CI = confidence interval

DISCUSSION

The aim of this explorative study was to investigate differences in anticoagulation control between men and women and between younger premenopausal and older postmenopausal women with a MHV on VKA treatment. The most important finding is the poor anticoagulation quality in younger women. This finding is based on several outcome parameters, among others time in therapeutic INR range (TTR), but also a higher percentage of INR results followed by a significant dose-adjustment (> 10%) and a lower percentage of INR results within therapeutic range.

The quality of anticoagulant control in premenopausal women using VKA has not yet been extensively investigated. Internationally, a TTR of 65% to 70% is recommended for optimal efficacy and safety of anticoagulant treatment in MHV patients[14,21]. We showed that in our study younger women have a much lower median TTR than the recommended 65%. This implies that over 50% of younger women do not reach a TTR of 65% as recommended by national and international guidelines[21]. It is known that factors such as younger age, female sex and high INR intensity are predictors of poorer anticoagulation control[14,24]. Other possible explanations for this lower TTR could be lifestyle factors (e.g. alcohol consumption and sport participation), and poor adherence to treatment[15]. We hypothesize that variation in the vitamin K dependent coagulation factor VII during the menstrual cycle[20] may also have an influence on anticoagulation control and perhaps this is an important factor in young women. However, as this study has an observational nature, the effect of the menstrual cycle and the impact of anticonception use on anticoagulation control should be topic of further research.

Although TTR was found to be lower in younger women compared to older women and age-matched men, based on our data there seemed to be no impact on clinical outcomes, as no differences between men and women in major clinical events such as major bleeding and ischemic stroke were found. With the hazard ratios of clinical events all around unity, the lower TTR in younger women was not associated with an increased risk of adverse events during follow-up, although the absolute number of major bleedings and ischemic stroke that occurred during the study period in this patient group was low. Therefore, the number of patients developing thromboembolism or major bleeding was too small to identify a clear relation between TTR and risk of these events. The similarity in patient outcome may also be explained by a lower a priori probability of major clinical events in a younger age group. In earlier literature however, also no differences in clinical events were found[12,14,24]. However, since poor TTR remains the most important predictor of long-term patient outcome during VKA therapy, methods to optimize anticoagulant control in young women should be a topic of future research.

There are some limitations of our study. First, this is an observational retrospective cohort study, and therefore we could not adjust for clinical information that was not recorded. In particular, the use of oral contraceptives is not routinely reported, with oral contraceptives also having their influence on the haemostatic system[25]. Second, no differences in major clinical events between younger and older women were found. Outcome and severity of all adverse events were registered by specialized physicians as part of usual medical care of the clinic. However, heavy menstrual bleeding is not recorded as bleeding complication on a regular basis. We did observe, however, more minor bleedings being reported by younger women (data not shown). This can even be an underestimation of the incidence of minor bleedings, as heavy menstrual bleeding is not actively been taken into account. It is known that women of reproductive age experience increased menstrual blood loss and change in

menstrual pattern while on oral anticoagulant therapy, with an incidence ranging from 22% to 65% in women treated with VKAs[26,27]. Therefore, in future studies, minor bleedings should be included as events. Third, this retrospective cohort study was conducted in a group of patients monitored by a Dutch Anticoagulation Clinic. A majority of patients however, as VKA treatment for the indication of a MHV is long-term treatment, will start with self-management after several months being monitored by an Anticoagulation Clinic. Fourth, the majority of patients used acenocoumarol. Acenocoumarol is the preferred VKA in the Netherlands and in Spain, while warfarin is the preferred drug in the United States, Canada and Italy[28]. Where acenocoumarol is a short acting VKA, warfarin and phenprocoumon are longer acting VKAs, hereby possible preventing fluctuations in coagulation factors, among others FVII[18,29]. However, warfarin is not registered for use in the Netherlands. It would be of interest to investigate whether younger women on a long-acting VKA have a higher and more stable TTR compared to a short-acting VKA. Unfortunately, the group of patients on a long-acting VKA was too small to do a sensitivity analysis in order to answer this question. Last, according to Dutch Primary Care Guidelines (NHG), the average age women reach their menopause in the Netherlands is around 51 years[30]. With the inclusion of an age group 16-45 years and an age group of 55 years and older, we tried to divide the study population in younger premenopausal and older postmenopausal women. However, as data about the menstrual state of women on VKAs at the time of their INR measurement is not routinely reported, it is not completely certain that all postmenopausal women are excluded from the younger age group and vice versa.

CONCLUSION

A poorer anticoagulation control was observed in younger women with MHV on VKA treatment compared to older women and age-matched men, based on several outcome parameters. Our study shows that >50% of these young women do not reach a TTR of 65% as recommended in current guidelines. Despite the lower TTR in younger women, no differences between men and women in major clinical events such as major bleeding and ischemic stroke were found. However, since poor TTR remains the most important predictor of long-term patient outcome during VKA therapy, methods to optimize anticoagulant control in young women should be a topic of future research.

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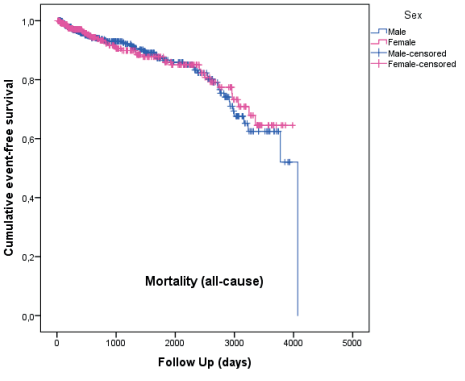
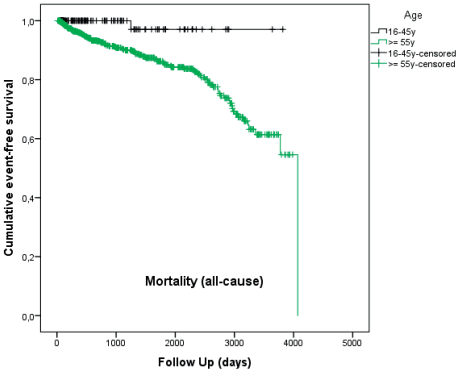


	Number at risk	Number of events	Log-Rank p-value
Male	696	58	
Female	481	41	0.899



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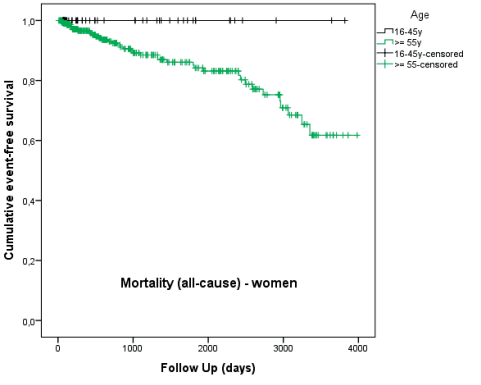
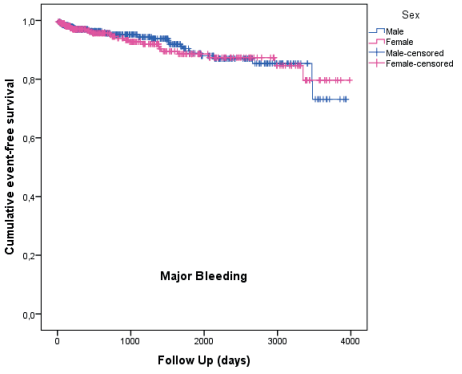
16-45y	156	1	
>= 55 y	1021	98	0.004



	Number at risk	Number of events	Log-Rank p-value
16-45y*	63	0	
>= 55 y*	418	41	0.027

*women only

Supplemental figure 1. Kaplan Meier curves of major clinical events



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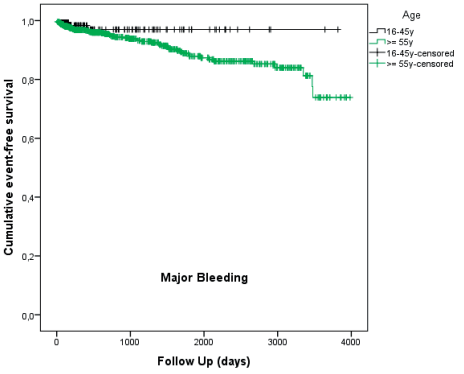
Male	696	37	
Female	481	27	0.824



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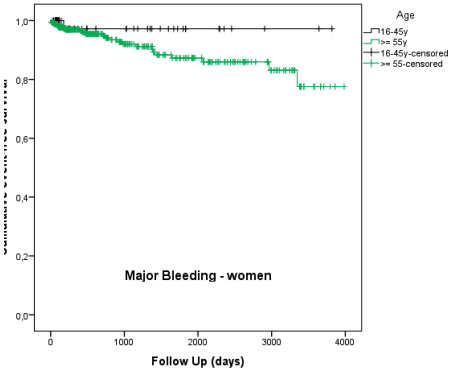
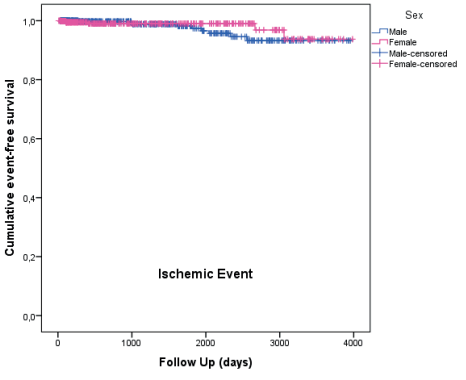
>= 55 y	1021	61	0.089



	Number at risk	Number of events	Log-Rank p-value
16-45y*	63	1	
>= 55 y*	418	26	0.161

*women only

Supplemental figure 1. Kaplan Meier curves of major clinical events (continued)



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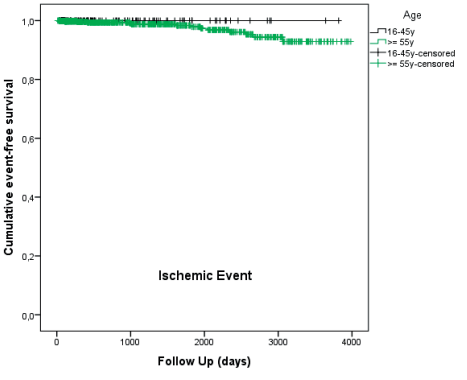
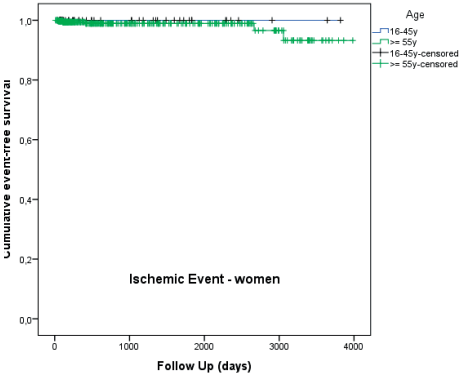
Male	696	9	
Female	481	5	0.610



	Number at risk	Number of events	Log-Rank p-value
16-45y	156	0	
>= 55 y	1021	14	0.234



	Number at risk	Number of events	Log-Rank p-value
16-45y*	63	0	
>= 55 y*	418	5	0.452

*women only

Supplemental figure 1. Kaplan Meier curves of major clinical events (continued)

PART III

General discussion & conclusion and summary

CHAPTER 9

General discussion

GENERAL DISCUSSION

Many clinical studies on patients with bleeding symptoms have consistently shown that no bleeding disorder can be diagnosed in 47% to 69% of patients, despite extensive laboratory evaluation.[1] These patients are classified as having Bleeding of Unknown / Undefined Cause (BUC). In a recent guideline, the European Hematology Association (EHA) defines BUC as *'Bleeder / bleeding of unknown cause (BUC) – a bleeding disorder fitting the definition of mild/moderate bleeding phenotype (but even possible severe in some rare cases) that cannot be associated to any hemostatic or genetic abnormality after extensive investigation with currently available techniques. It cannot be excluded that under this provisional category future novel techniques may identify new disease entities.'*[2] One of the major aims of this thesis was to investigate the diagnostic process and management of patients with BUC, by gaining more insight in the role of global screening tests in the diagnostic process and in the optimal treatment strategy for these patients.

This thesis consists of two parts, the first part focusing on diagnosis and management of BUC patients, with focus on female patients, as the majority of patients being referred for bleeding symptoms is female (>80%). The second part focuses on female specific health issues in haemostasis and thrombosis. Women have a higher chance of manifest bleeding due to female specific bleeding events including menstrual cycle and childbirth. In women, hemostasis is influenced by physiological changes in hormone status associated with the menstrual cycle, pregnancy, and hormonal therapy, which can lead to changes in coagulation, causing either a hypo- or a hypercoagulable state. The main findings of the studies described in this thesis will be discussed and interpreted in this final chapter. In addition, recommendations for improving patient care for patients with BUC, and women with bleeding or thrombotic problems more specifically, will be made. Finally, suggestions for further research will be addressed.

PART I - The Clinical Relevance and Significance of New Diagnostic Options in patients with an unexplained bleeding tendency – Results from the 'Crescendo-study'

Bleeding of unknown cause: distinguishing physiological from pathological bleeding, role of bleeding assessment tools

The first step in the diagnostic process of patients with a bleeding tendency is to distinguish pathological and therefore clinically relevant bleeding from physiological bleeding, e.g. bleeding considered as normal for a healthy subject. Healthy subjects frequently report minor bleedings, with epistaxis, easy bruising and prolonged bleeding after tooth extraction being most common.[1,3] These bleeding symptoms may have clear underlying causes as

trauma or mucosal lesions. In addition, in the healthy female population menorrhagia is a commonly reported bleeding symptom, with a reported prevalence from 30 to 47%.[3,4]

Obtaining a detailed bleeding history is an important part of haemostatic evaluation to determine whether a patient has a clinical relevant bleeding tendency, is at increased risk of (major) bleeding in response to invasive procedures, should be evaluated by laboratory evaluation or referred to a haematologist. Over the last years, several bleeding assessment tools (BATs), translating experienced bleeding symptoms into an objective bleeding score (BS), have been developed as screening tools to facilitate objective documentation of bleeding symptoms and severity of bleeding in patients with a bleeding tendency.[5,6]

Although BATs are important diagnostic tools, in both chapter 2 and chapter 3 we report that 44 – 55% of BUC patients presented with a normal BS. In 21 – 52% of patients with a mild bleeding disorder (MBD) a normal BS was found.[7] In a recently published study by Gebhart et al., two BATs (Vicenza BAT and the ISTH-BAT) were analysed in a large group of adult patients with a mild to moderate bleeding tendency. The authors showed that both BATs had a low ability to discriminate patients with a diagnosis of an established bleeding disorder from patients with BUC.[8] Also, in several other studies no differences in bleeding scores have been found between patients with and without an established MBD, and patients with BUC seem clinically indistinguishable from those with a known MBD as von Willebrand disease (VWD).[8-13] Therefore, a BAT should only serve as one of many diagnostic tools available in the diagnostic work up of patients with a bleeding tendency.

A recently published consensus report of the EHA[2] states that the aim of the BAT is not to demonstrate a strict correlation between an identifiable bleeding disorder and a bleeding score, but to identify those individuals that are at significant risk of future bleeding. Two major studies showed that a high BS has been demonstrated to be highly predictive of post-surgical bleeding for patients with various types of VWD[14] and inherited platelet disorders (PD).[15] Furthermore Relke et al.,[16] found that a higher BS was associated with a significantly higher risk of future spontaneous bleeding events in BUC patients. The usefulness of a BAT to predict bleeding in VWD was previously also shown by Federici et al.[14] Thus, an useful application of BATs could be the ability to identify (BUC) patients who are more likely to bleed excessively during invasive procedures, surgery and childbirth.

Pathophysiology and the role of global haemostatic testing in the diagnostic process of BUC

Currently, it is only possible to speculate on the pathophysiological mechanisms of BUC. Because the nature, sites, and severity of bleeding seems similar to patients with MBDs, including those with VWD and low von Willebrand factor levels (low VWF)[11], it is stated that patients with BUC are likely to have a primary haemostasis disorder, such as an unknown platelet function defect. Increased fibrinolytic activity, which cannot be assessed with current analytical tools, could also be the cause of BUC. Furthermore, the pathogenesis of BUC could be multifactorial, with an accumulation of several subtle impairments in primary

and / or secondary haemostatic factors and / or fibrinolytic factors, possibly even without a decrease below normal cut-off levels.[17]

The role of patient characteristics in the diagnostic process of BUC

Age and comorbidities of patients could also be of influence in the diagnostic process of BUC. Patients with mild bleeding symptoms may not often suffer from bleeding symptoms in daily life. Problems may occur after a haemostatic challenge, with a first challenge possibly occurring at later age. It has been shown that several haemostatic factors increase with age.[18] This might explain that no abnormalities are found in this possible 'older' patient group at time of analysis. In addition, the role of comorbidities can be more pronounced in an older population, for example the influence of age and comorbidities on skin and vessels, possibly causing easy bruising or perioperative bleeding.[19]

In this thesis, we investigated the role of global haemostasis tests in BUC patients, by means of rotational thromboelastometry (ROTEM), the thrombin generation (TG) assay, and the plasma clot lysis assay. ROTEM, a viscoelastic whole blood assay of haemostasis introduced as a point-of-care test (POCT) device, is increasingly used in the assessment of coagulation and bleeding in the emergency and perioperative setting, hereby guiding management.[20] The diagnostic value of ROTEM in haemostatic evaluation of patients with a bleeding tendency is currently unknown. In chapter 3 we report that in our BUC patient cohort, ROTEM variables of BUC patients are within reference ranges and do not differ from healthy controls and patients in whom a MBD was diagnosed.[7] Our results are in line with those described by Wieland Greguare-Sander et al.,[21] who also aimed to investigate the sensitivity of ROTEM for diagnosis of MBD. Only a weak association between the presence of a MBD and ROTEM variables was found, and all variables were within the established reference ranges. Based on our own findings, we came to the same conclusion as Wieland Greguare-Sander et al., namely that there is no support for the additive value of ROTEM in screening and diagnosing patients with a (mild) bleeding tendency.

However, ROTEM has been shown to be of additive value in the optimization of management of bleeding patients in different clinical situations. In chapter 4, we report that in trauma and surgical patients acquired FIBTEM clot firmness parameters correlate well with final clot stiffness as measured by the FIBTEM assay as well as to fibrinogen levels as measured by the Clauss assay, a more time consuming method for determination of fibrinogen levels. This means that early FIBTEM parameters can be used to evaluate fibrinogen concentrations in acute medical settings, hereby reducing the time to diagnosis of coagulopathy, and guiding rapid clinical management, a finding confirmed in several other studies.[22,23]

The primary aim of POCT in the intensive care and surgical setting, however, should be an improvement in clinically relevant outcomes, such as reduction in bleeding-related morbidity and mortality. This has not yet been proven in settings apart from the management of perioperative bleeding in cardiac surgery and liver transplantation.[24-27] In obstetric situations,

most relevant being postpartum haemorrhage (PPH), Collins et al., have reported on the value of fibrinogen and FIBTEM as predictors of severe PPH among women with persistent PPH.[28] Amgalan et al., recently systematically reviewed studies that investigated TEG/ROTEM use in pregnancy and peripartum. The authors conclude that ROTEM-guided algorithms have been developed to guide transfusion therapy in PPH, but that these algorithms have not consistently shown advantages over conventional approaches.[29] Large multi-center randomized clinical trials should be designed and performed in order to determine whether ROTEM not only predicts PPH and guides transfusion, but also improves clinical outcome of women with PPH. An important aim of these trials should be to unravel mechanisms of excessive bleeding based on individual patient characteristics during PPH.

Thrombin is a key protein in haemostasis and the measurement of thrombin generation (TG) has been applied regularly to investigate bleeding risk in patients with a bleeding disorder.[30-33] We report that, parameters measured by the TG assay did not differ between patients with BUC and healthy controls besides a significant longer lag time in BUC patients. With these results, we confirm results of several other studies.[34-36] Contradictory, a prolonged lag time was previously reported in patients with venous thrombosis.[37] However, it is also shown that women using oral contraceptives have a shorter lag time and patients on VKA-therapy have a prolonged lag time, based on a population based, prospective, observational, single-center cohort study (The Gutenberg Health Study), that included 5000 patients.[38] This might indicate that a delayed clot formation contributes to bleeding in patients with BUC. Our findings, however, are in contrast with recently published data, in which all TG variables in BUC patients were found to be significantly different from healthy controls, with a prolonged lagtime (min), a decreased peak thrombin (nmol/L), a prolonged time to peak (TTP, min) and a decreased area under the curve (AUC, nmol/L x min) found in BUC patients. One explanation could be the larger sample size of this specific study, with 382 patients with BUC being included.[12] Also, the TG assay used was from a different manufacturer than the assay that was used in our study. The authors conclude that TG can be used in situations where traditional clotting tests such as aPTT and PT fail to differentiate. For the use of TG in patients with BUC, standardization of test conditions is of eminent importance in order to implement this assay into routine clinical practice, as is shown that TG is greatly influenced by preanalytical conditions.[39]

Based on our study, also no evidence was found supporting hyperfibrinolysis as a cause of bleeding in BUC patients. This finding was, however, in contrast with recently published data, in which an increased susceptibility to clot lysis in patients with BUC was found.[12] In this study, clot formation rate and clot lysis time were able to distinguish BUC patients from healthy controls. In contrast, we found that clot lysis time was significantly prolonged in patients with BUC, with and without addition of potato carboxypeptidase inhibitor (PCI, an inhibitor of activated TAFI), compared to healthy controls. These findings were opposite to the study hypothesis but in line with other published data about fibrinolysis in BUC patients

as well as in women with unexplained menorrhagia.[40-42] In these studies, in which 95 patients with an undiagnosed mild bleeding tendency and 97 women with menorrhagia were included, patients with bleeding symptoms did not have faster clot lysis than controls. These and our findings support a trend towards reduced fibrinolysis in BUC. Until date, there is no good explanation for these findings.

In our study, we only investigated fibrinolysis by means of the plasma clot lysis assay and by measuring α-2-antiplasmin (α2-AP) levels in patients, the latter as part of routine care. Fibrinolysis inhibitor protein deficiencies such as plasminogen activator inhibitor 1 (PAI-1) and α2-AP have been associated with a bleeding phenotype.[43,44,45] Although deficiencies of these proteins effect the global fibrinolytic process, as should be detected by a global clot lysis assay, these deficiencies could be minor or borderline-normal, not influencing global fibrinolysis. Therefore, it would be of interest to measure individual fibrinolysis proteins in our BUC patient cohort, such as PAI-1 or TAFI. Currently, only one Dutch Hemophilia Treatment Center (HTC) tests for PAI-1 deficiency and hyperfibrinolysis. These patients are included in the Rare Bleeding Disorders in the Netherlands (RBiN) study, which investigates patients with hereditary rare bleeding disorders. Until November 2019, 263 patients were included in the RBiN study, with 14 (5%) patients having a PAI-1 deficiency and 14 (5%) patients with hyperfibrinolysis.[46]

Based on the findings described above, we conclude that currently there is no role for global haemostasis tests (e.g. thromboelastometry, thrombin generation or plasma clot lysis time) in the diagnostic process of patients with a mild to moderate bleeding tendency without an established bleeding disorder, classified as patients with BUC. This conclusion is based on conflicting results regarding the utility of these tests in BUC patients, as described in this thesis and literature.

Management of BUC

Only limited evidence is available regarding the most adequate treatment for BUC patients during haemostatic challenges such as surgery or childbirth. In chapter 2, a cohort of BUC patients is described, who received several different treatment regimens or no treatment before intervention. Most patients received tranexamic acid with or without desmopressin. Still, not all BUC patients in our cohort received haemostatic treatment perioperative or during childbirth. An incidence of 25% of major bleeding and a striking incidence of 46% of postpartum haemorrhage in BUC patients without haemostatic treatment was found.

In the patients that receive perioperative tranexamic acid and/or desmopressin bleeding complications were seen in only 14% of BUC patients, meaning 86% did not experienced any bleeding complications during or after surgery. As far as we know, only a study by Obaji et al.,[47] and a recently published study by MacDonald et al.,[34] reported on outcome of haemostatic challenges in BUC patients treated with TXA and / or desmopressin. Both studies found

similar results, with tranexamic acid and / or desmopressin given as prophylactic perioperative haemostatic treatment being effective in 90% of cases.[47]

The International Working Group (IWG) established by the EHA developed a series of guidelines on MBDs and states that BUC patients should be treated as if they have a true bleeding disorder. Therefore antifibrinolytics, such as tranexamic acid, or desmopressin should be used and drugs interfering with haemostasis should be avoided.[2] Because no laboratory abnormalities are identified in patients with BUC, the pathogenesis of bleeding is unknown. Bleeding in this group may be caused by a higher fibrinolytic activity, which however we could not confirm in our study (chapter 3), or can be multifactorial, caused by an accumulation of subtle impairments. This may explain why, at least in part, bleeding in these patients seems to be controlled by pro-hemostatic medication such as desmopressin and TXA, and therefore is recommended as empirical treatment to prevent bleeding during surgery and after trauma. Desmopressin and tranexamic acid are both considered to be safe treatment options in different medical situations, with both desmopressin and tranexamic acid having mostly mild side effects reducing blood loss and transfusion requirements with no significant thrombotic adverse effects found in several placebo-controlled studies.[48-51] We therefore recommend prophylactic perioperative treatment with tranexamic acid in all patients with BUC, with proactive and early addition of desmopressin when necessary.

Recommendations for further research in BUC

Based on obvious conflicting results in literature, both on the value of BATs as well as on the pathophysiological mechanisms of BUC, additional studies on patients with BUC are required. First aim of these studies should be the identification of 'true' bleeders: patients that are at risk for major bleeding complications during future haemostatic challenges, versus 'normal' bleeders. In addition, the most optimal treatment strategy for BUC patients should be part of future studies, preferably large prospective trials, as evidence regarding the most optimal treatment strategy for haemostatic challenges in BUC patients is still scarce.

Also, there is a need for studies focusing on the underlying pathophysiological mechanisms of BUC. To date, there seems no role for global haemostasis tests in BUC. Fibrin clot structure however, is the major determinant of mechanical stability and resistance to lysis of a clot.[52] Investigation of fibrin clot structure has yet no role in routine diagnostic haemostatic testing. Limited studies have been conducted investigating clot structure in bleeding disorders, in contrast to thrombotic disorders. The few studies that investigated clot structure in established bleeding disorders all show that different factor deficiencies cause clots with a reduced fiber density and relatively thick fibers. Clots from haemophiliacs have been shown to have a decreased clot stiffness.[53] It is also shown that clots formed without factor IX in a Haemophilia B model, are composed of thicker fibrin fibers than those formed in the presence of factor IX.[54] Factor XI deficient patients with an increased bleeding tendency have a reduced fibrin network density, compared to controls and non-bleeders.[55]

Finally, clots in the presence of factor XIII form significantly thinner fibers and have a higher density of fibers compared to those without factor XIII.[56] Based on these findings, although challenging, investigating clot structure could be of additive value in the diagnostic process of BUC patients.

In the near future, advanced techniques such as Next Generation Sequencing (NGS)-based gene panels [57,58] or Whole Exome Sequencing (WES) including both platelet and non-platelet-related genes[59] may lead to discoveries of novel genes playing a role in haemostasis or bleeding disorders. However, translating these results into daily practice will provide a next challenge due to multi-interpretable and uncomprehensive findings such as variants of unknown significance (VUS) and incidental findings, such as variants associated with predisposition to cancer, e.g. RUNX1 mutations with predisposition to acute myeloid leukemia. This, making a multidisciplinary approach for interpretation of the genetic results into clinical practice, in combination with thorough medical-ethical consideration obligatory. Before implementation of genetic screening in the standard diagnostic workup of patients with a bleeding tendency or BUC, prospective studies are needed, with large patient numbers and cost-benefit analysis.

PART II - Female specific health issues in Haemostasis and Thrombosis

Diagnosis of bleeding disorders in women

It has been shown that there is a female predominance in patients with mild bleeding disorders, with over 80% of patients being female.[7,9,34,60] Obviously, women have a higher chance of manifest bleeding due to menstrual cycle and childbirth. However, MacDonald et al.[34] report that in their cohort of BUC patients, elevated bleeding scores persist in women when female specific bleeding issues (being menorrhagia and postpartum haemorrhage (PPH)) are removed from the bleeding score. Mauer et al., who studied bleeding symptoms in a healthy population, report an equal number of self-reported experienced bleeding symptoms by men and women, when removing female specific bleeding symptoms. However, after analyzing individual symptoms by means of logistic regression, it was found that easy bruising and venipuncture bruising was more prevalent in women. Anatomical rather than haemostatic causes or a different perception of the experienced symptoms might contribute to this difference. Also, the role of female hormones on skin and vessels is largely unknown. However, it is unlikely that this high percentage of women with BUC is solely based on an altered perception or undiagnosed anatomical reasons.

Postpartum haemorrhage in women with BUC, established bleeding disorders and without diagnosed bleeding disorders

There are several important obstetric causes and risk factors for PPH, with uterine atony, a retained placenta and lower genital tract trauma being most prevalent.[61,62] In chapter 5, we report on a cohort of women referred for haemostatic analysis after severe PPH (≥

2000ml blood loss). In 23% of included women, a mild bleeding disorder could be diagnosed after haemostatic evaluation. We did not find any significant differences in the presence of obstetrical causes and risk factors between patients with and without a diagnosed bleeding disorder.[63] Therefore, an obstetrical cause or the presence of obstetrical risk factors in women with severe PPH does not automatically rule out an underlying haemostatic defect and a multifactorial cause of PPH is assumable. Our finding of 23% of women being diagnosed with a mild bleeding disorder was in contrast to the only study published investigating postpartum haemorrhage as predictor of inherited bleeding disorders. In this study by Kadir et al., only 1 of 50 included women was identified to have type 2 VWD.[64] It is not completely clear what diagnostic criteria were used for diagnosing VWD in this study and if low VWF levels were found in any of these women. In conclusion: even the presence of anatomical abnormalities and / or obstetric causes for severe PPH is no reason for abandoning haemostatic evaluation in these women. Recognizing and diagnosing underlying bleeding disorders in women with PPH as well as menorrhagia has important clinical implications. It enables adequate treatment, for example with antifibrinolytic agents (TXA) and / or desmopressin, and awareness of an increased bleeding risk during (major) surgical intervention or delivery later in life.

In a recently published international, randomised, double-blind placebo-controlled trial (the WOMAN trial), 20.060 women with PPH were enrolled.[50] It was found that tranexamic acid reduces death due to bleeding in women with post-partum haemorrhage with no adverse effects. When used as treatment for PPH, it is recommend that TXA is given as soon as possible after bleeding onset. Furthermore, it is known that previous PPH results in a three times higher risk of recurrent PPH during a subsequent delivery.[65-67] In chapter 2 we reported on patients diagnosed with BUC and outcome of surgical procedures and deliveries. We described that only 8% of women with BUC received haemostatic treatment during childbirth, namely TXA in 2 of the 24 women. Forty-two percent of these women had major PPH in their medical history. In this cohort, a striking incidence of 11/24 (46%) of major PPH was found. Currently, guidelines do not recommend to proactively administer tranexamic acid to women with PPH in their medical history. In addition, administration of haemostatic therapy is based on factor levels during third trimester of pregnancy, usually rising within or above normal range in women with mild VWD1 of low VWF levels, indicating adequate haemostasis before delivery. In chapter 5, we described a cohort of women with severe PPH (n= 85) referred for haemostatic evaluation. Of these women, less than half (44%) received tranexamic acid at time of ongoing blood loss, even if they had a history of PPH. The administration of TXA was not associated with the year of delivery and no increase in administration of TXA over the years was seen. In a recently published systematic review, Punt et al. describe that both women with VWD receiving prophylaxis during childbirth as well as untreated women with VWD, based on 'normalization' of their clotting factor levels, are at a higher risk of primary and secondary PPH compared to the general population.[68] The authors suggest that more

aggressive obstetric management with liberal use of tranexamic acid to prevent primary as well as secondary PPH could be required. Based on our findings, we also recommend a proactive third stage of labour with a combination of early administration of uterotonics and additional haemostatic therapy, preferably tranexamic acid, especially in women with a history of PPH.

Chauleur et al. report that even mild haemostatic abnormalities are independently associated with a significantly increased risk for severe PPH, which include slightly decreased levels of fibrinogen, and low von Willebrand factor (VWF) levels.[17] This is confirmed by our finding in chapter 5 that about half of the women referred for haemostatic evaluation after severe PPH was diagnosed with low VWF or mild VWD type 1 (9/16, 56%). This might indicate that, although there is a physiological increase in plasma FVIII and VWF levels during pregnancy[66,69], some women with low VWF apparently still are at risk for PPH. This finding is in line with recently published data.[70] Lavin et al. found an increased incidence of PPH in women with low VWF levels, with 63.5% of included women reported excess bleeding at time of delivery. Interestingly, following low VWF diagnosis and enrolment in the LoVIC study, 32 women underwent 38 pregnancies, with VWF-levels all corrected within or above normal nonpregnant VWF-levels by third trimester. Still 21% of these women experienced excessive bleeding during delivery.[70] This is in line with earlier data from women with an established bleeding disorder including haemophilia carriers and women with VWD, reported by Stoof et al., and more recently by Zwagemaker et al.[66,71] Both studies recommend administration of prophylactic treatment aiming at higher factor levels before delivery (FVIII and VWF level >150 U/ml), closer to reached factor levels in third trimester found in healthy pregnant women. Based on these and our data target peak level >150 U/ml VWF level could be considered during the peripartum period, in addition to early administration of TXA. However, further studies are needed to investigate the mechanisms underlying PPH in women with low VWF levels and optimal strategies for clinical management of these women.

Also, a more multifactorial cause of PPH is suggested in literature. In PPH, antifibrinolytic agents, such as tranexamic acid, have been proven to be of value during PPH[51], suggesting that bleeding might be caused by a higher fibrinolytic activity. Also, it is shown that even a mild decreased level of fibrinogen is associated with a significantly increased risk for severe PPH.[17] Therefore, a disturbed fibrin clot structure might, at least in part, explain the bleeding risk in women who thus far have not been diagnosed with a bleeding disorder. Therefore, fibrin clot structure of women with and without PPH was investigated in this thesis. Unfortunately, this study was designed as a pilot study, and therefore the number of participants and data was insufficient to detect any but the largest differences. Although a trend was seen in clot characteristics such as the mean fiber diameter, number of thick and thin fibers, fiber density, and number and size of pores, and these findings were similar to findings described in several previous studies investigating clot structure in patients with

coagulation factor deficiencies[53-56], no significant difference were found between women with and without PPH.

Sex related differences in haemostatic variables and the need for female-specific reference ranges

Previously reported data consistently show a difference in the levels of several coagulation factors between men and women.[72-76] In women, hemostasis is influenced by physiological changes in hormone status associated with the menstrual cycle, pregnancy, and hormonal therapy such as contraceptives or hormone replacement therapy. In case of hormonal therapy, influences on coagulation depends in particular on estrogen levels.[77] These hormonal influences can lead to an increased risk of venous thromboembolism (VTE) due to altered levels of clotting factors and an acquired resistance to activated protein C.

Currently, routine haemostatic reference ranges used in the laboratory are commonly based on a group of healthy male or mixed population blood donors.[78] In chapter 7, we report on a cohort of 197 women with a pregnancy complicated by preeclampsia, in whom thrombophilia investigation was performed. We showed that over 40% of women would be falsely classified as having one or more abnormal thrombophilic factors, such as decreased protein C, protein S or antithrombin, using routine reference ranges (RRR). Significantly more abnormal results were found in women after an uncomplicated pregnancy (n=61) and preeclampsia (n=197) when using standard RRR (54% versus 74% respectively) than when using women-specific reference ranges (WRR)(11% and 33% respectively), calculated based on a group of 55 women with a recent uncomplicated pregnancy.[79] We chose to use the Reference Value Advisor software for determining these women-specific reference ranges, which is guided by the IFCC-CLSI recommendations[80] and permits evaluation and transformation of data distributions and computation of reference ranges with the corresponding confidence intervals.

This finding indicates that women-specific reference ranges should be used for the interpretation of haemostatic variables as false classification can have major consequence for future pregnancies and other situations with an increased thrombosis risk throughout life. A critical note however, is that a wide range of values for coagulation and clotting factors has been reported in both healthy individuals as well as patients with a diagnosed bleeding disorder.[7,81] A considerable overlap between healthy individuals and patients with mild bleeding disorders was found. In addition, a wide inter-individual and intra-individual variability of these coagulation and clotting factors has been reported. Therefore, when only minor differences are seen between reference values from women and men, it is important there is a clear clinical relevance, and hereby clinical consequence, of these small differences. Another important limitation of using women-specific reference ranges, is that hormone levels vary throughout the menstrual cycle and throughout life. In addition, these levels can be influ-

enced by the use of exogenous hormones, for example the oral contraceptive pill. Therefore, complete information about the patient (e.g. day of menstrual cycle of menopausal status) is necessary in order to adequately interpret laboratory results.

Anticoagulation control in young women with mechanical heart valves treated with VKAs
Mechanical heart valves (MHV) are highly thrombogenic leading to a substantial high risk of thrombosis and systemic embolism[82-84]. For this reason, long-term management with oral anticoagulant therapy with vitamin K antagonists (VKAs) is recommended[84]. Predictors of poor anticoagulation control of vitamin K antagonist (VKA) treatment include young age and female sex. These lead to a higher risk of complications during VKA therapy.[85-87] In chapter 8, difference in anticoagulation control between pre- and postmenopausal women and age-matched men was investigated in patients with a MHV using VKAs. A poorer anticoagulation control was observed in younger women with MHV on VKA treatment compared to older women and age-matched men, based on several outcome parameters. Our study showed that more than half of these young women do not reach an adequate level of anticoagulation (TTR > 65%) as recommended in current guidelines.

During the menstrual cycle, changes are observed in levels of VWF, fibrinogen, and activated factor VII (FVII),[88-90] that could be the effect of hormonal changes, with lowest levels being measured during menstruation and early follicular phase.[89] The level of coagulation FVII is also the most important determinant of INR stability during treatment with VKAs, which is strongly related to the half-life of the used VKA (acenocoumarol versus warfarin).[91,92] Therefore, we hypothesize that the poorer quality of anticoagulation control in young women could be influenced by the cyclic variation of FVII, and the role of FVII in INR stability. In our study however, we had no information about hormonal status of these women, e.g. use of oral contraceptives or other forms of contraception. In addition, this finding did not have any clinical consequences with hazard ratios of clinical events all around unity, concluding that in our study, this lower TTR in younger women is not associated with an increased risk of adverse events during follow-up. This finding is most probably based on the low samples size of our study, with a low number of young women being included (n=63), and therefore a low absolute number of major bleedings and ischemic strokes that occurred during the study period. Also, younger patients have a lower a priori chance for developing ischemic complications. The observed difference between younger and older women and younger women and younger men is not completely understood. Various factors could be of influence on this poorer anticoagulation controls, e.g. lifestyle factors (e.g. alcohol consumption and sport participation), and poor adherence to treatment.[86] This poor adherence to treatment could be caused by increased menstrual blood loss and change in menstrual pattern while on oral anticoagulant therapy. The incidence of heavy menstrual blood loss ranges from 22% to 65% in women treated with VKAs.[93,94] Since VKA is the preferred anticoagulant in patients

with MHV, optimization of VKA treatment for specifically young premenopausal women is of great importance.

Our results are important because patients with a mechanical heart valve are still treated with a VKA, because dabigatran, one of the direct oral anticoagulants (DOACs) which are preferred anticoagulant treatment in patients with atrial fibrillation or venous thrombosis, was shown to be inferior to warfarin in patients with MHVs, both in terms of efficacy (e.g. ischemic stroke risk) and safety (e.g. bleeding risk).[95] So, as recommended by both European and American guidelines [84,96,97], patients with MHVs should be treated with VKAs. However, VKAs require frequent coagulation monitoring and dose adjustments to ensure that the INR remains within the therapeutic range. Until date, only one other study was conducted in 10 patients with MHVs using rivaroxaban. The authors showed that rivaroxaban was safe and effective in low risk patients with mechanical aortic heart valves, with neither thromboembolic nor bleeding events during the observation period of 6 months. They however also state that these results need justification in larger studies in this specific patient population.[98] Recent studies have shown that dabigatran, rivaroxaban and apixaban are less effective than warfarin at inhibiting MHV-induced thrombin generation.[99,100] They conclude that, although these static in vitro models may not reflect the complexities of MHV-induced thrombosis in humans, VKAs are likely to remain the standard care for prevention of MHV-induced thrombosis. However, studies about DOAC therapy in this specific patient group are ongoing.

Recommendations for further research in women specific health issues in thrombosis and haemostasis

Although the influence of sex on coagulation factors is well known, the female predominance of patients referred for a bleeding tendency is not completely understood. In addition, the role of sex hormones in bleeding, being clearer in thrombotic disorders, is largely unknown. In recent years, several articles have been published in the field of thrombosis and haemostasis, highlighting the effects of sex differences. However, most of these studies encompass cardiovascular and thrombotic research projects. In the scientific field of bleeding attention is focused on female-specific bleeding problems (e.g. menorrhagia and PPH), but differences between men and women in the pathophysiology of bleeding are still unknown. Future studies should therefore focus on these pathophysiological mechanisms, thereby unravelling the role of sex related hormones in bleeding, followed by optimization of gender-specific treatment strategies.

It is shown that BATs can have a role in identifying patients at risk for more excessive bleeding during invasive procedures, surgery and childbirth. In PPH however, it has been showed that a BAT used as a screening tool contributing to the identification of women with an increased risk for PPH lacks discriminative power.[101] Therefore, trials investigating mechanisms of excessive bleeding, other than the known obstetrical causes and risk factors, based on individual patient characteristics during PPH are necessary.

We recommend future studies within a larger population of young women using VKAs, so that better conclusions can be drawn with regard to clinical consequences of the poorer anticoagulation control in these women. In these studies, the hormonal status of young women should be part of the investigation of anticoagulation control. We recommend the inclusion of women using acenocoumarol and compare them with women using phenprocoumon, which causes less FVII fluctuations, women using acenocoumarol and oral contraceptives (no hormonal fluctuations) and postmenopausal women. In addition, our findings should be confirmed in comparative studies including self-monitoring patients, as can be hypothesized that most young patients will switch to self-monitoring when having a lifelong indication for VKA treatment.

In conclusion, the studies described in this thesis provide new insights in the diagnostic process and management of patients with inherited and acquired bleeding and thrombotic disorders with special emphasis on women. In general, further research should be aimed at unravelling the underlying pathophysiological mechanism of bleeding of unknown cause, as conflicting results are reported in literature, and therefore most optimal management strategy is still unknown. Furthermore, there should be specific attention for sex differences in coagulation, hereby optimizing clinical trials and women-specific patient care.

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CHAPTER 10

Summary & samenvatting

SUMMARY

Many clinical studies on patients with (mostly mild) bleeding symptoms have consistently shown that, after wide-ranging laboratory evaluation, in between 47% and 69% of patients no bleeding disorder can be diagnosed. These patients are classified as having Bleeding of Unknown / Undefined Cause (BUC). The European Hematology Association (EHA) defines BUC as *'Bleeder / bleeding of unknown cause (BUC) – a bleeding disorder fitting the definition of mild/moderate bleeding phenotype (but even possible severe in some rare cases) that cannot be associated to any hemostatic or genetic abnormality after extensive investigation with currently available techniques. It cannot be excluded that under this provisional category future novel techniques may identify new disease entities.'* Patients with BUC seem clinically indistinguishable from those with a known mild bleeding disorder (MBD). Currently, it is only possible to speculate on the pathophysiological mechanism of BUC.

In several cohort studies the majority of patients with BUC is female (around 80%). Bleeding issues in women include heavy menstrual bleeding (HMB), ovulation bleeding, excessive and prolonged bleeding after miscarriage, and primary and secondary postpartum haemorrhage (PPH). It is also known that coagulation factor levels differ between men and women, and some coagulation factors show a cyclic variation. The influence of these differences on hemostasis are still largely unknown, but could play a role in both bleeding and thrombotic complications in women.

In this thesis, the results of several studies focusing on diagnosis and management of patients with bleeding of unknown cause (BUC) and female specific health issues in thrombosis and haemostasis, are reported.

In **chapter 1**, a short introduction to this thesis is presented. The definition of bleeding of unknown cause (BUC) is introduced, which affects 47-69% of patients referred for a bleeding tendency to specialized haematology outpatient clinics. For these patients, even after extensive haemostatic laboratory investigation, diagnostic uncertainty remains. Also, optimal management for prevention and treatment of bleeding in BUC patients is unclear, as only limited evidence on effective and adequate management strategy is available. Global screening tests, e.g. rotational thromboelastometry, thrombin generation test, and plasma clot lysis time are introduced, which assess the overall haemostatic potential, and therefore may be of value in screening and diagnosis of BUC. Furthermore, several female specific health issues in thrombosis and haemostasis are introduced in this chapter.

In **part I**, the current management of patients with BUC undergoing a surgical procedure and / or delivery is evaluated with the aim to investigate outcomes of recommended management strategies for BUC patients. In addition, the role of global screening tests (rotational thromboelastometry, thrombin generation test, plasma clot lysis assay) in the diagnostic work-up of patients with a bleeding tendency is evaluated.

In **chapter 2**, the management and outcome of haemostatic challenges in patients referred to our tertiary outpatient clinic with a bleeding tendency (n = 380) was retrospectively evaluated, as only limited evidence on effective and adequate management strategy for BUC patients is currently available. In BUC patients (n=228), administration of desmopressin and / or TXA was most often recommended as management during surgery or other interventions. Despite these measures, 19% (10/52) of surgical procedures was complicated by major bleeding in this specific patient group. In women classified as having BUC, only 8% (2/24) received prophylactic haemostatic treatment peripartum, with a striking incidence of 46% (11/24) of major PPH. Further investigation of the most optimal treatment strategy for BUC patients, and a proactive attitude during surgery and delivery with early additional haemostatic therapy, is recommended to prevent major bleeding and major PPH.

As it has been suggested that coagulation tests that assess the overall haemostatic potential may be of additive value in screening for and diagnosis of bleeding disorders, in **chapter 3**, we reported our prospective cohort study. In this study, 181 patients referred for analysis of a bleeding tendency to our tertiary outpatient clinic and 76 healthy controls were included, we investigated the diagnostic value of global haemostasis tests in these patients. No major differences were found in thromboelastometry variables in patients with BUC compared to healthy controls. The thrombin generation test showed a significantly increased lag time in patients with BUC, possible indicating prolonged clot formation. Some BUC patients had a significantly prolonged clot lysis time, indicative of an impaired or decreased fibrinolysis.

We described correlations between fibrinogen concentration measured by the Clauss assay and FIBTEM clot firmness parameters (one of the variables of rotational thromboelastometry) in different patient groups and healthy individuals in **chapter 4.** During acute settings accompanied by major blood loss, it is important for clinicians that recent fibrinogen-levels are available as soon as possible, in order to guide adequate management. In this study, a total of 116 patients of whom both ROTEM and Clauss assay results were available were included, in addition to 75 healthy subjects. The majority of patients underwent cardiac surgery (41%), experienced bleeding or other trauma (30%), or underwent a liver transplant procedure (11%). Early FIBTEM clot firmness parameters correlated well with a final clot stiffness as measured by the FIBTEM assay and with fibrinogen concentration as measured by the Clauss assay. This means that early FIBTEM parameters can be used to evaluate fibrinogen concentrations in acute medical settings, and to guide clinical management.

In **part II** of this thesis, focusing on **Female specific health issues in haemostasis and thrombosis**, we reported on a cohort of women with severe PPH referred to our outpatient clinic for haemostatic evaluation in **chapter 5**. Postpartum haemorrhage (PPH), defined as ≥500 ml blood loss within 24 hours postpartum, is the major cause of maternal death worldwide. Previous studies revealed that haemostatic abnormalities are independently associated with a significantly increased risk for severe PPH (≥2000ml). In this study, the value of haemostatic

evaluation in women with severe PPH was explored. In total, 85 women with severe PPH were included. In 23% (n=16) a mild bleeding disorder (MBD) was diagnosed, including low Von Willebrand factor (Low VWF 8/16), platelet function disorders (PFD 5/16), BUC (2/16) and Von Willebrand Disease type 1 (1/16). The diagnosis of a MBD was independent of obstetrical causes or risk factors for PPH, which implies that severe PPH can be the first clinical symptom of an inherited or acquired bleeding disorder, and haemostatic evaluation after severe PPH is recommended.

In **chapter 6**, we performed a small pilot study where the fibrin clot structure of women with and without major PPH was investigated by means of Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy. A disturbed fibrin clot structure might, at least in part, explain the bleeding risk in women, as clot structure affects clot characteristics such as resistance to lysis and mechanical deformation. Ten patients with severe PPH and 5 controls with an uncomplicated delivery were included ≥3 months postpartum. Number of fibrin fibers, fiber diameter, fiber density, pore size and number of pores were analyzed. Fibrin clots acquired from women with severe PPH tended to have a reduced density, thicker fibers, and a larger pore size, compared to clots from women with an uncomplicated delivery. These observations suggest that the clots of women with severe PPH possibly exhibit an abnormal structure with an increased susceptibility to fibrinolysis that may contribute to severe blood loss after delivery. However, this study was designed as a pilot study, and the number of participants and data was insufficient to detect any but the largest differences. Further research, in order to confirm our findings, is recommended.

It is known that levels of several coagulation factors are generally lower in women than in men. In **chapter 7,** we studied the effect of using women-specific reference ranges for thrombophilia-related haemostatic variables. Women-specific reference ranges based on a group of 55 healthy women were calculated. When using these women-specific reference ranges, compared to routine reference ranges based on healthy male of mixed-population blood donors, significantly more normal results with regard to thrombophilia-parameters were found, in healthy women as well as in women diagnosed with preeclampsia. This indicates that women-specific reference ranges can be helpful for interpretation of haemostatic variables as misclassification of the laboratory findings in women can have major consequences throughout life.

In **chapter 8,** we focused on the other side of the haemostatic balance, the thrombotic complications. In this chapter, the results of the investigation of anticoagulation control in premenopausal women with mechanical heart valves (MHVs) using vitamin K antagonists (VKAs) are described. The most important determinant of INR stability during treatment with VKAs is the level of coagulation factor VII (FVII). As in premenopausal women levels of coagulation factors during the menstrual cycle may vary, this may be associated with a lower quality of anticoagulation control. In order to explore this hypothesis, 1177 MHV patients were included, of whom 41% were female. Thirteen percent of patients was younger than

45 years during treatment with VKA. Younger women were less well anticoagulated as evidenced by a significantly lower time in therapeutic range (TTR) and cross-sectional proportion (CSP)(64.5% and 51.9%) compared to older women (71.5% and 71.0%; both $p<0.05$) and significantly lower TTR compared to young men (70.1%, $p<0.05$). Hazard ratios for clinical events were all around unity. As patients with MHV are at significant risk of complications, optimization of VKA therapy in young women is of great importance.

In **chapter 9 and 10**, the findings of this thesis are summarized and discussed in the view of other recent studies, and results are put in a clinical perspective. In addition, implications of the findings for daily patient care as well as future research are given.

SAMENVATTING

Veel klinische studies die patiënten met (veelal milde) bloedingsklachten betroffen hebben consequent aangetoond dat, ook na uitgebreide laboratoriumevaluatie, bij 47% tot 69% van de deze patiënten geen stollingsstoornis kan worden gediagnosticeerd. Bloedingen bij deze patiënten worden geclassificeerd als 'Bloeding met onbekende / niet-gedefinieerde oorzaak' (Bleeding of Unknown / Undefined Cause (BUC)). De European Hematology Association (EHA) definieert BUC als volgt: *stollingsstoornis met bloedingsklachten die passen binnen de definitie van een mild / matig bloedingsfenotype (maar in zeldzame gevallen ook met een ernstig bloedingsfenotype) welke niet kan worden verklaard door middel van een bekende stollingsstoornis of genetische afwijkingen na uitgebreid onderzoek middels de momenteel beschikbare technieken. Het kan niet worden uitgesloten dat binnen deze voorlopige categorie toekomstige nieuwe technieken mogelijk nieuwe ziekte-entiteiten kunnen identificeren.*' Patiënten met BUC zijn klinisch nauwelijks te onderscheiden van patiënten met een bekende milde stollingsstoornis (mild bleeding disorder, MBD). Met de huidige kennis is het alleen mogelijk te speculeren over het onderliggende pathofysiologische mechanisme van BUC.

Uit verschillende cohortstudies blijkt dat de meerderheid van patiënten met BUC vrouw is (rond de 80%). Het is bekend dat de hoogte van verschillende stollingsfactoren verschilt tussen man en vrouw. Ook laten sommige stollingsfactoren een variatie zien gedurende de menstruale cyclus. De rol van deze verschillen tussen man en vrouw en de cyclische variatie van stollingsfactoren is nog niet geheel duidelijk, maar zou een rol kunnen spelen in stolling gerelateerde gezondheidsproblemen bij vrouwen.

In dit proefschrift worden de resultaten beschreven van verschillende studies die gericht zijn op de diagnose en behandeling van patiënten met BUC en vrouwspecifieke stolling gerelateerde gezondheidsproblemen.

In **hoofdstuk 1** wordt een korte introductie van dit proefschrift gegeven. De definitie van 'bleeding of unknown cause' wordt geïntroduceerd, welke van toepassing is op 47-69% van de patiënten die in verband met een verhoogde bloedingsneiging verwezen zijn naar gespecialiseerde hematologische klinieken. Zelfs na uitgebreid laboratoriumonderzoek is de diagnose bij deze groep patiënten niet altijd te achterhalen. Daarnaast is er nog veel onduidelijkheid over de preventie en optimale behandeling van bloedingen bij deze patiënten. Op dit moment is er slechts beperkt wetenschappelijk bewijs voor de meest effectieve behandelstrategie. In dit hoofdstuk worden ook globale stollingstesten beschreven, zoals rotatie tromboelastometrie (ROTEM®), de trombine generatie test en de plasma clot lysis assay. Aangezien deze testen de gehele stolling in beeld brengen, in plaats van slechts individuele stollingsfactoren, zouden deze mogelijk van waarde kunnen zijn bij het diagnosticeren van patiënten met BUC. Ook worden verschillende vrouwspecifieke stolling gerelateerde problemen beschreven.

In **deel I** van dit proefschrift wordt de huidige behandeling van patiënten met BUC en de uitkomsten van verschillende chirurgische procedures en bevallingen in kaart gebracht. Ook wordt de rol van globale stollingstesten (rotatie tromboelastometrie (ROTEM®), de trombine generatie test en de plasma clot lysis assay) in het diagnostisch proces van patiënten met een bloedingsneiging geëvalueerd.

In **hoofdstuk 2** hebben wij retrospectief de behandeling en uitkomsten van verschillende chirurgische ingrepen en bevallingen van patiënten die eerder naar onze tertiaire kliniek waren verwezen in verband met een bloedingsneiging (n = 380) onderzocht. Dit omdat er momenteel slechts beperkte informatie beschikbaar is over wat de meest effectieve en adequate behandelstrategie voor BUC-patiënten is. In onze studie werd bij BUC-patiënten (n = 228) de toediening van desmopressine (DDAVP) en / of tranexaminezuur het meeste aanbevolen als behandeling vooraf of tijdens operaties en andere interventies. Ondanks deze maatregelen werd in ons studiecohort 19% (10/52) van de chirurgische ingrepen gecompliceerd door een ernstig bloedingen. Verder kreeg van de vrouwen met BUC slechts 8% (2/24) hemostatische behandeling voorafgaand aan een bevalling, waarbij in totaal 46% (11/24) van de bevallingen gecompliceerd werd door een ernstige postpartum bloeding. Om ernstige bloedingen te voorkomen, zal er verder onderzoek naar de meest optimale behandelstrategie voor BUC-patiënten gedaan moeten worden. Ook wordt een proactieve houding tijdens chirurgische ingrepen en bevallingen, door middel van vroegtijdig toedienen van aanvullende hemostatische therapie, aanbevolen.

Omdat stollingstesten die de gehele stolling in beeld brengen, mogelijk van toegevoegde waarde kunnen zijn bij het screenen op en diagnosticeren van bloedingsstoornissen bij patiënten met BUC, beschrijven we in **hoofdstuk 3** onze prospectieve cohortstudie. In deze studie hebben wij 181 patiënten met een bloedingsneiging, verwezen naar onze tertiaire polikliniek voor diagnostiek naar mogelijke stollingsstoornissen, en 76 gezonde controles geïncludeerd. De diagnostische (toegevoegde) waarde van globale stollingstesten bij BUC-patiënten werd onderzocht. Er werden geen grote verschillen gevonden in de onderzochte tromboelastometrie variabelen tussen BUC-patiënten en gezonde controles. De trombine generatie test liet een significant vertraagde initiatie van de stolling (lagtime) zien bij patiënten met BUC, waardoor een stolsel mogelijk trager gevormd wordt. Ook hadden BUC-patiënten een significant verlengde clot lysis tijd in vergelijking met gezonde controles, wat kan wijzen op vertraagde fibrinolyse.

Tijdens acute medische situaties die gepaard gaan met grote hoeveelheden bloedverlies, is het van belang dat de meest recente fibrinogeenspiegels zo snel mogelijk beschikbaar zijn om de beste behandeling te kunnen starten. In **hoofdstuk 4** beschrijven we de correlaties tussen twee verschillende methoden om de fibrinogeenconcentratie te meten, namelijk de veelgebruikte Clauss-methode en de parameters van de FIBTEM (een van de variabelen van de ROTEM). In deze studie werden in totaal 116 patiënten en 75 gezonde proefpersonen geïncludeerd van wie zowel ROTEM- als Clauss-methode resultaten beschikbaar waren.

De meerderheid van de geïncludeerde patiënten onderging een hartoperatie (41%), werd behandeld in verband met een bloeding of trauma (30%) of onderging een levertransplantatie (11%). De vroege FIBTEM-parameters correleerden goed met de uiteindelijk gemeten latere FIBTEM-parameter clot firmness en met fibrinogeenconcentraties zoals gemeten met de Clauss methode. Dit betekent dat vroege FIBTEM-parameters gebruikt kunnen worden om fibrinogeenconcentraties vroeg in acute medische situaties te bepalen en hiermee het klinisch beleid te sturen.

In **deel II** van dit proefschrift, gericht op **vrouwspecifieke gezondheidsproblemen rondom hemostase en trombose**, beschrijven we een cohort van vrouwen met ernstige postpartum bloedingen die na de bevalling naar onze polikliniek verwezen werden voor hemostatische evaluatie in **hoofdstuk 5.** Postpartum bloedingen (PPH), gedefinieerd als ≥ 500 ml bloedverlies binnen 24 uur na de bevalling, zijn wereldwijd de belangrijkste oorzaak van moedersterfte. Eerdere studies toonden aan dat stollingsafwijkingen onafhankelijk geassocieerd zijn met een significant verhoogd risico op ernstige PPH (≥ 2000 ml bloedverlies binnen 24 uur na de bevalling). In onze studie onderzochten wij de waarde van stollingsdiagnostiek bij vrouwen met ernstige PPH onderzocht. In totaal werden 85 vrouwen met ernstige PPH geïncludeerd. Bij 23% (n = 16) van deze vrouwen werd een milde bloedingsstoornis (MBD) aangetoond, waaronder lage Von Willebrand-factor waarden (low VWF 8/16), plaatjesfunctiestoornissen (PFD 5/16), BUC (2/16) en Von Willebrand ziekte type 1 (1/16). De diagnose van een MBD was onafhankelijk van onderliggend aanwezige obstetrische oorzaken of risicofactoren voor PPH, wat impliceert dat ernstige PPH het eerste klinische symptoom kan zijn van een erfelijke of verworven stollingsstoornis. Hemostatische evaluatie na ernstige PPH wordt daarom aanbevolen.

In **hoofdstuk 6** beschrijven we een kleine pilotstudie waarin de structuur van bloedstolsels van vrouwen met en zonder ernstige postpartum bloedingen werd onderzocht door middel van Scanning Electron Microscopie (SEM) en Confocal Laser Scanning Microscopie. Een verstoorde fibrine structuur in een stolsel zou, gedeeltelijk, het bloedingsrisico bij vrouwen kunnen verklaren. De structuur van het bloedstolsel kan namelijk de gevoeligheid voor fibrinolyse en mechanische vervorming beïnvloeden. Tien patiënten met ernstige PPH en 5 controles met een ongecompliceerde bevalling werden ≥ 3 maanden na de bevalling geïncludeerd. Het aantal fibrinedraden, de diameter van de fibrinedraden, de dichtheid van het fibrine netwerk, de poriegrootte van het fibrine netwerk en het aantal poriën werden geanalyseerd. In de stolsels van vrouwen met ernstige PPH lijkt sprake van een verminderde dichtheid van fibrinedraden, dikkere fibrinedraden en een grotere poriegrootte, vergeleken met stolsels van vrouwen met een ongecompliceerde bevalling. Deze bevindingen suggereren dat de stolsels van vrouwen met ernstige PPH mogelijk een abnormale structuur hebben, met hierdoor een verhoogde gevoeligheid voor fibrinolyse, wat kan bijdragen aan ernstige bloedverlies na de bevalling. Deze studie is echter opgezet als pilotstudie, waardoor het aantal deelnemers en de verzamelde gegevens onvoldoende was om grote verschil-

lende te detecteren. Verder onderzoek om onze bevindingen te bevestigen, wordt daarom aanbevolen.

Het is algemeen bekend dat de levels van verschillende stollingsfactoren over het algemeen lager zijn bij vrouwen dan bij mannen. Momenteel worden routine referentiewaarden van stollingsfactoren berekend op basis van gezonde mannelijke of gemengde bloeddonoren. Een verkeerde interpretatie van laboratoriumbevindingen bij vrouwen kan grote gevolgen kan hebben voor het beleid ten aanzien van chirurgische ingrepen of bevallingen. In **hoofdstuk 7** hebben we het effect van het gebruik van vrouwspecifieke referentiewaarden voor trombofilie factoren onderzocht. Vrouwspecifieke referentiewaarden werden berekend op basis van een groep van 55 gezonde jonge vrouwen. De resultaten van gemeten trombofilie factoren lagen significant vaker binnen de vrouwspecifieke referentiewaarden dan binnen de routine referentiewaarden. Dit was zowel het geval bij gezonde vrouwen als bij vrouwen met pre-eclampsie tijdens de zwangerschap. Dit geeft aan dat vrouwspecifieke referentiewaarden nuttig kunnen zijn voor de interpretatie van hemostatische variabelen.

In **hoofdstuk 8** richten we ons op trombotische complicaties. In dit hoofdstuk worden de resultaten beschreven van ons onderzoek naar de kwaliteit van antistolling bij premenopauzale vrouwen met een mechanische kunstklep (MHV) die behandeld worden met een vitamine K-antagonist (VKA). De belangrijkste determinant van de INR-stabiliteit tijdens de behandeling met VKAs is het niveau van stollingsfactor VII (FVII). Aangezien de niveaus van stollingsfactoren tijdens de menstruatiecyclus bij premenopauzale vrouwen kunnen variëren, kan dit verband houden met een verminderde kwaliteit van antistollingstherapie. Om deze hypothese te onderzoeken, werden 1177 patiënten met een MHV geïncludeerd, waaronder 41% vrouwen. Dertien procent van deze patiënten was tijdens de behandeling met VKA jonger dan 45 jaar. Jongere vrouwen hadden een lagere kwaliteit van antistollingstherapie, zoals blijkt uit een lagere time in therapeutic range (TTR) vergeleken met oudere vrouwen (64,5% versus 71,5%). Ook de cross-sectional proportion (CSP) was significant lager bij jonge vrouwen ten opzichte van ouderen vrouwen (51,9% versus 71,0%). Hazard ratio's voor klinische complicaties waren allen rond 1. Aangezien patiënten met een MHV een aanzienlijk risico lopen op complicaties, is optimalisatie van VKA-therapie bij jonge vrouwen van groot belang.

In **hoofdstuk 9 en 10** worden de bevindingen van dit proefschrift samengevat en bediscussieerd in het licht van andere recent gepubliceerde studies, en worden de gevonden resultaten in klinisch perspectief geplaatst. Ook worden de mogelijke betekenissen van onze studies voor de dagelijkse patiëntenzorg en toekomstig onderzoek beschreven.

PART IV

Appendices

LIST OF PUBLICATIONS

MANUSCRIPTS RELATED TO THIS THESIS

Veen CSB, Biedermann JS, Witkam WCAM, Leebeek FWG, Kruip MJHA, Anticoagulation control in premenopausal women with mechanical heart valves using vitamin K antagonists: room for improvement, *Manuscript submitted*

Veen CSB, Huisman EJ, Romano LGR, Schipaanboord CWA, Cnossen MH, de Maat MPM, Leebeek FWG, Kruip MJHA, Outcome of surgical interventions and deliveries in patients with bleeding of unknown cause (BUC): an observational study, *Manuscript accepted Thrombosis and Haemostasis*

Daraei A, Pieters M, de Lange Z, Baker SR, Litvinov RI, **Veen CSB**, de Maat MPM, Weisel JW, Ariens RAS, Guthold M, Automated fiber diameter and porosity measurements of fibrin clots in Scanning Electron Microscopy images, *Manuscript submitted*

Veen CSB, Donkel SJ, Nagaswami C, Weisel JW, Kruip MJHA, de Maat MPM, Fibrin clot structure in severe postpartum haemorrhage, *Manuscript submitted*

De Vries JJ, **Veen CSB**, Snoek CJM, Kruip MJHA, de Maat MPM, FIBTEM Clot Firmness paramaters correlate well with the fibrinogen concentration measured by the Clauss assay in patients and healthy subjects, *Scand J Clin Lab Invest,* 2020 Sep;14:1-6

Veen CSB, Huisman EJ, Cnossen MH, Kom-Gortat R, Rijken DC, Leebeek FWG, de Maat MPM, Kruip MJHA, Evaluation of thromboelastometry, thrombin generation and plasma clot lysis time in patients with bleeding of unknown cause: a prospective cohort study, *Haemophilia,* 2020 May;26(3):e106-e115

Veen CSB, van der Reijken IS, Jansen AJG, Schipaanboord CWA, Visser W, de Maat MPM, Leebeek FWG, Duvekot JJ, Kruip MJHA, Severe postpartum haemorrhage as first presenting symptom of an inherited bleeding disorder, *Haemophilia,* 2019 Nov;25(6):1051-1058

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MANUSCRIPTS NOT RELATED TO THIS THESIS

Veen CSB, Bikker G, van der Straaten H, De kunst van het kijken – Uw diagnose? *Ned Tijdschr Hematol,* 2016;13:153-154

Kordasti S, Marsh J, Al-Khan S, Jiang J, Smith A, Mohamedali A, Abellan PP, **Veen CSB**, Constantini B, Kulasekararaj AG, Benson-Quarm N, Seidl T, Mian SA, Farzaneh F, Mufti GJ, Functional characterization of CD4+ T cells in aplastic anemia, *Blood*, 2012;119(9):2033-2043.

SCIENTIFIC SESSIONS

2019 **13th Dutch Hematology Conference, Papendal, The Netherlands**
Oral presentation: *'Anticoagulation control in premenopausal women with mechanical heart valves using vitamin K antagonists: room for improvement'*

2018 **2nd European Congress on Thrombosis and Haemostasis, Marseille, France**
Oral presentation: *'Severe postpartum hemorrhage as a first presenting symptom of a bleeding disorder'*
Poster presentation: *'Reduced clot firmness may contribute to bleeding phenotype in patients with mild bleeding disorders'*

2018 **25th Fibrionogen and 3rd FXIII Workshop, Winston-Salem, United States**
Oral presentation: *'Abnormal fibrin clot structure in women who experienced postpartum hemorrhage'*

2018 **COEUR PhD Course Sex and Gender in Cardiovascular Research, Rotterdam, The Netherlands**
Oral presentation (invited speaker): '*Clinical differences between sexes in bleeding and thrombosis'*

2017 **XXVI Congress of the International Society on Thrombosis and Haemostasis (ISTH) and 63rd Annual SSC Meeting, Berlin, Germany**
Poster presentation: *'Thrombophilia: Women-specific reference ranges may prevent overdiagnosis'*
Poster presentation: *'Anticoagulation control in premenopausal women with mechanical heart valves using vitamin K-antagonist: room for improvement'*

2017 **7th International Symposium on Women's Health Issues in Thrombosis and Haemostasis, Barcelona, Spain**
Poster presentation: *'Thrombophilia: Women-specific reference ranges may prevent overdiagnosis'*

2016 **1st European Congress on Thrombosis and Haemostasis, The Hague, The Netherlands**
Oral presentation: *'The necessity of refining the diagnostic evaluation of patients with a bleeding tendency not explained by current routine laboratory testing'*

AWARDS AND PRIZES

2018 **Young Investigator Award**
2nd European Congress on Thrombosis and Haemostasis, Marseille, France

2017 **International Society on Thrombosis and Haemostasis (ISTH) Training Fellowship**
International Society on Thrombosis and Haemostasis (ISTH)

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Tot slot, lieve kleine **Nora**. Alles valt eigenlijk in het niet en lijkt zo onbelangrijk vergeleken bij het geluk dat wij ervaren sinds jij in ons leven bent. Zo klein als je bent, zo groot is onze dankbaarheid voor jouw komst. We genieten iedere dag opnieuw van jou en al je ontwikkelingen! Groei onbezorgd op, blijf je vrolijke zelf en doe datgene waar je blij van wordt!

CURRICULUM VITAE / ABOUT THE AUTHOR

Caroline S.B. Veen (inmiddels officieel Damhuis-Veen) werd geboren op 28 juni 1986 te Naarden. Na het behalen van haar vwo-diploma in 2005 aan het Oostvaarders College in Almere werd zij in eerste instantie uitgeloot voor de studie Geneeskunde. In 2006 behaalde zij haar propedeuse Algemene Gezondheidswetenschappen aan de Vrije Universiteit te Amsterdam. In datzelfde jaar mocht zij starten met de studie Geneeskunde, eveneens aan de Vrije Universiteit te Amsterdam. Haar wetenschappelijke stage deed zij gedurende zes maanden op de afdeling Haematological Medicine van het King's College Hospital te Londen, alwaar zij het voorkomen van verschillende subgroepen dendritische cellen bij hematologische maligniteiten onderzocht. Na haar semiarts stage op de afdeling Hematologie van het VU medisch centrum, behaalde zij begin 2013 haar masterdiploma. Na het behalen van haar artsendiploma werkte zij als arts-assistent (ANIOS) op de afdelingen Interne Geneeskunde van het Zaans Medisch Centrum te Zaandam en Spoedeisende Hulp van het Sint Jansdal Ziekenhuis te Harderwijk.

In 2015 begon zij aan haar promotieonderzoek op de afdeling Hematologie van het Erasmus MC te Rotterdam onder supervisie van dr. M.J.H.A. Kruip en prof. dr. F.W.G. Leebeek. De resultaten van dit promotieonderzoek kunt u teruglezen in dit proefschrift.

In 2018 heeft zij de overstap gemaakt naar de eerstelijnsgeneeskunde en gedurende 10 maanden in het verpleeghuis gewerkt, waarna zij gestart is met de opleiding tot huisarts aan het Leids Universitair Medisch Centrum. Tijdens deze opleiding bemerkte zij een toenemende passie voor specifiek de oudere patiëntenpopulatie en heeft daarom besloten terug te keren naar het verpleeghuis, waar zij tot op heden met heel veel plezier werkzaam is. Sinds maart 2021 is zij als specialist ouderengeneeskunde in opleiding werkzaam bij de Frankelandgroep te Schiedam.

Waar 2020 voor velen misschien een jaar was om snel te vergeten, kende 2020 voor Caroline twee grote hoogtepunten. Op 11 juni 2020 mocht zij dochtertje Nora verwelkomen en 2 november van datzelfde jaar gaven zij en haar echtgenoot elkaar het ja-woord.

PHD PORTFOLIO

Name PhD student: Caroline S.B. Veen
Erasmus MC Department: Hematology
Research School: COEUR

PhD period: May 2015 – November 2018
Promotor: Prof. dr. F.W.G. Leebeek
Co-promotor: Dr. M.J.H.A. Kruip

	Year	Workload (ECTS)
PhD Training		
General academic skills		
CPO Course (Centre for Patient Oriented Research)	2015	0.3
Working with Endnote – Erasmus MC Medical Library	2015	0.5
Systematic Literature Retrieval – Erasmus MC Medical Library	2015	0.5
Open Clinica Course	2015	0.3
Good Clinical Practice (eBROK)	2015	1.0
Biomedical English Writing and Communication	2017	3.0
Research Integrity	2017	0.3
Research skills		
Biostatistical Methods I: Basic Principles (NIHES)	2015	5.7
In-depth courses (e.g. Research School, Medical Training)		
2x NVTH annual AIO course on Haemostasis and Thrombosis	2016 & 2018	2.0
COEUR Course on Cardiovascular Medicine	2015	1.5
COEUR Research Seminar Hemostasis and Thrombosis in Children	2016	0.3
COEUR Course Intensive Care	2017	0.3
COEUR Course on Sex and Gender in Cardiovascular Research	2018	0.3
(Inter)national scientific presentations and conferences		
Oral presentations		
European Congress on Thrombosis and Haemostasis (ECTH), The Hague, The Netherlands	2016	1.0
COEUR Course on Sex and Gender in Cardiovascular Research (invited)	2018	1.0
Trombosestichting, Ambassadeursavond (invited)	2018	1.0
Fibrinogen and FXIII Workshop, Winston-Salem, US	2018	1.0
European Congress on Thrombosis and Haemostasis (ECTH), Marseille, France	2018	1.0
Dutch Hematology Congress, Papendal, The Netherlands	2019	1.0
Poster presentations		
Symposium on Women's Health Issues in Thrombosis and Hemostasis, Barcelona, Spain	2017	0.3
2x International Society on Thrombosis and Haemostasis, Berlin, Germany	2017	0.3

	Year	Workload (ECTS)
European Congress on Thrombosis and Haemostasis (ECTH), Marseille, France	2018	0.3
International conferences		
European Congress on Thrombosis and Haemostasis (ECTH), The Hague, The Netherlands	2016	0.9
Symposium on Women's Health Issues in Thrombosis and Hemostasis, Barcelona Spain	2017	0.9
International Society on Thrombosis and Haemostasis, Berlin, Germany	2017	1.8
Fibrinogen and FXIII Workshop, Winston-Salem, US	2018	1.2
European Congress on Thrombosis and Haemostasis (ECTH), Marseille, France	2018	0.9
National conferences		
2x Dutch Hematology Congress, Papendal, The Netherlands	2016 & 2019	1.2
AMSTOL Symposium	2016	0.3
Maastricht Summer School on Thrombin Generation	2016	0.6
NVTH Symposium	2018	0.6
Seminars and workshops		
3x COEUR PhD day	2015, 2016, 2018	0.9
Thrombin Generation Training UMC Maastricht & Stago	2015	0.3
ROTEM User Meeting	2015	0.3
2x Local training for Hematologists	2015 & 2017	0.6
Hematology PhD training		
16x Work discussions and journal clubs Erasmus MC	2015-2018	8.0
Teaching activities		
Lecturing		
5x Coagulation lecture for nurses	2015-2018	0.5
Supervising		
Review 2nd year medical students (coagulation course)	2015	0.5
Master thesis Celesta Schipaanboord, medical student	2016	1.0
Gradution project Ilona Edelijn, final year biomedical laboratory sciences	2016-2017	1.0
Master thesis Ilse Slotboom, medical student	2017	1.0
Master thesis Irene van der Reijken, medical student	2017-2018	1.0
Master thesis Willemijn Witkam, medical student	2017-2018	1.0
Gradution project Regina Kom-Gortat, final year biomedical laboratory sciences	2018	1.0

	Year	Workload (ECTS)
Other		
Personal coaching 1st and 2nd year medical students, 5 students in total	2016-2018	-
2x Clinical lecture for staff of the diagnostic haemostasis laboratory	2017 & 2018	0.2
Other		
ISTH Training Fellowship	2017	-
Laser Scanning Confocal Microscopy - Introduction Course	2017	0.3
Podcast recording Journal of Applied Laboratory Medicine	2018	-
Total		48.9

ECTS = European Credit Transfer and Accumulation System (1 ECTS represents 28 hours)