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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ALAT	Alanine amino transferase
ANOVA	Analysis of Variance
APTT	Activated Partial Thromboplastin Time
CI	Confidence Interval
F	Factor
GCP	Good Clinical Practice
HIN	Haemophilia in the Netherlands
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ISTH(SSC)-BAT	International Society on Thrombosis and Haemostasis (Scientific and Standardization Committee)- Bleeding Assessment Tool
METC	Medical research ethics committee (in Dutch: medisch ethische toetsings commissie)
NHA	Novel Haemostasis Assay
NVHP	Netherlands Haemophilia Society; (in Dutch: Nederlandse Vereniging van Hemofilie patiënten)
PAI	Plasminogen Activator Inhibitor
PFA	Platelet Function Analyser
Pro-RBDD	Prospective Rare Bleeding Disorder Data register
PT	Prothrombin Time
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TiN	Thrombopathy in the Netherlands
TPA	Tissue-type Plasminogen Activity
TT	Thrombin Time

TSH	Thyroid Stimulating Hormone
VWF	Von Willebrand Factor
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
WES	Whole Exome Sequencing
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: Rare bleeding disorders (deficiency of fibrinogen, factor II, V, V&VIII, VII, X, XI, XIII, α2-antiplasmin or plasminogen activator inhibitor 1) are not well defined with respect to their clinical phenotype, laboratory phenotype en genotype. At present, little is known about their clinical presentation, bleeding scores, bleeding episodes, health-related quality of life, laboratory parameters, genetics and current treatment. There are large differences in bleeding tendency and weak correlations with the level of factor deficiencies. Therefore, it is essential to perform thorough research in patients with rare bleeding disorders and perform laboratory and genetic tests, to seek explanations for the variety in clinical phenotype.

Objective: The purpose of the RBiN study is to describe the epidemiology, bleeding tendency, laboratory parameters, quality of life and genetics of all known patients in the Netherlands with rare bleeding disorders. In addition, the study aims to examine the relationship between clinical phenotype, laboratory phenotype and genotype.

Study design: cross-sectional multicenter observational study.

Study population: all patients registered in Dutch Haemophilia Treatment Centers with known disorders of the coagulation factors fibrinogen, factor II, V, V & VIII, VII, X, XI, XIII, α2-antiplasmin and plasminogen activator inhibitor type 1, aged 1 years and older.

Main study parameters/endpoints:

Description of the clinical phenotype, laboratory phenotype, genotype and quality of life.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: participating patients will be invited for one visit to their treatment center in order to draw blood, take a saliva sample and perform questionnaires. This will take approximately 40 to 120 minutes. Since the population of patients with RBDs is very small it is important to include all patients, also minors (children <18 years), in the study (around one third of known patients are minors). Therefore, this study may be regarded as group-related. The risk associated with participation is negligible.

1. BACKGROUND

Research of inherited bleeding disorders traditionally focuses on haemophilia because of the relatively 'common' presentation combined with a dramatic clinical picture. Haemophilia A - deficiency of coagulation factor (F) VIII - , and haemophilia B - deficiency of FIX – occur at an incidence of 1 in 10,000 and 1 in 60,000 live births, respectively [1]. In contrast, the rare bleeding disorders affecting coagulation factors fibrinogen, factor II, V, V&VIII, VII, X, XI and XIII, α2-antiplasmin and plasminogen activator inhibitor type 1 (PAI-1) are 10 to 200 times less frequent than haemophilia, with a general population prevalence estimated at 1:500,000 up to 1 per two million (Table 1~~Table 1~~) [1, 2]. Worldwide 16,000 patients have been registered though this is likely a serious underestimation of the actual patient number [3].

Their extreme rarity, clinical heterogeneity, difficulty in recognising affected patients, limiting laboratory assays and challenges in collecting longitudinal data have led to a gap in knowledge on all aspects of these rare disorders, and possibly to an underreporting of less severe cases. It is therefore imperative to bundle research efforts at a national (or even international) level to gain insight, as few centers have the possibility to follow and manage a consistent number of patients.

Table 1 General population prevalence of homozygous coagulation factor disorders

Disorder	Full name and variants (also known as)	Estimated general population prevalence homozygosity
Coagulation factor disorders		
- I	Fibrinogen disorders • Afibrinogenemia • Hypofibrinogenemia • Dysfibrinogenemia	1:1 million [1, 2]
- II	• Prothrombin deficiency (hypoprothrombinemia) • Dysprothrombinemia	1:2 million [1, 2]
- III	Platelet tissue factor: deficiency assumed to be incompatible with life [4]	
- IV	Calcium: no longer considered a 'mere' coagulation factor	
- V	Factor V deficiency (proaccelerin deficiency, labile factor deficiency, Owren's disease, parahemophilia)	1:1 million [1, 2]
- V & VIII	Combined FV & FVIII deficiency	1:1-2 million [2]
- VI	Activated form of FV, no longer recognised as a coagulation factor on its own	
- VII	Factor VII deficiency (proconvertin deficiency, Alexander's disease)	1:500,000 [1, 2]
- VIII	Haemophilia A (classic haemophilia)	1:10,000
- IX	Haemophilia B	1:60,000
- X	Factor X deficiency (thrombokinase deficiency	1:500,000-1 million [1, 2, 5]

	,Stuart-Prower factor deficiency)	
- XI	Factor XI deficiency (plasma thromboplastin antecedent deficiency, Rosenthal syndrome, haemophilia C)	1:1 million [1, 2]
- XII	Factor XII deficiency (Hageman factor deficiency; not associated with bleeding tendency)	
- XIII	Factor XIII deficiency (fibrin stabilising factor deficiency)	1:1-2 million [1, 2]
Fibrinolysis disorders		
- α2-Antiplasmin	α2-Antiplasmin deficiency (Miyasato disease)	Unknown, 40 cases reported worldwide [6, 7]
- Plasminogen activator inhibitor type 1	Plasminogen activator inhibitor type 1 deficiency	Unknown, few cases reported worldwide [8, 9]

1.1. Epidemiology of the rare bleeding disorders

Rare bleeding disorders are scarce. General population prevalence for homozygosity is estimated at 1:500,000 for factor VII deficiency up to 1 per million or 1 per two million for the disorders of fibrinogen, and the factor II, V, V&VIII, X, XI, and XIII deficiencies (Table 1~~Table 1~~) [1, 2]. These estimates originate from case descriptions, case series or at best registry studies, undertaken several decades ago. At present, these estimated prevalences are the best we have, and they are generally accepted. In general, data from registries and case series are prone to underestimating the true population prevalence because of problems with capturing the entire population and underreporting of less severe cases [3]. Reported prevalences of patients with clinically significant deficiencies (factor levels of 10% or less) registered in comprehensive registries in Iran, Italy and the United Kingdom are of the same magnitude as the population prevalence estimates [10].

1.2. Clinical manifestations

Patients affected by rare bleeding disorders have a wide spectrum of clinical presentations that varies from a mild or moderate bleeding tendency to potentially serious or life-threatening haemorrhages [11].

Occasionally, the interplay with other inherited coagulation disorders such as platelet defects, von Willebrand disease and other coagulation factor deficiencies may give a more severe bleeding tendency [12-18].

There is a heterogeneous association between coagulation factor activity level and clinical bleeding severity in the different rare bleeding disorders. The coagulation factor activity levels that are necessary for patients to remain asymptomatic are [19]:

- Fibrinogen: > 100 mg/dL;
- FV: 12%;
- FV & VIII: 43%;
- FVII: 25%;
- FX: 56%;
- FXI: 26%;
- FXIII: 31%

Coagulation factor activity levels that corresponded with spontaneous major bleeding were [19]:

- Fibrinogen: undetectable levels;
- FV: undetectable levels;
- FV & VIII: 15%;
- FVII: 8%;
- FX: 10%;
- FXI: 25%;
- FXIII: undetectable

For the fibrinolysis disorders, there are no data available on the factor activity levels necessary to remain asymptomatic. Available data on patients with rare bleeding disorders are based on clinical records, which will predominantly include patients presenting with more severe bleeding symptoms. This generates a high risk of ascertainment bias, with a possible overestimation of the severity of bleeding in both homozygous and heterozygous individuals at group level.

Fibrinogen disorders

Afibrinogenemia (defined as fibrinogen <200 mg/L) is inherited in an autosomal recessive pattern. Patients with congenital afibrinogenemia suffer from a lifelong severe bleeding, with haematomas, haematemesis, melaena, or umbilical cord bleeding manifesting in the newborn period in severe cases [20]. Internal bleeding and spontaneous splenic rupture are common features, as is severe bleeding after minor trauma, easy bruising and gingival haemorrhage. In a study from 1971 that described all known case-reports of afibrinogenemia up to that time (n=23) 52% of patients reported umbilical bleeding, 70% severe bleeding after minor traumata and 13% central nervous system bleeding [21]. Arterial and venous thromboembolic complications were also recorded in some cases, as well as recurrent miscarriage [22].

Dysfibrinogenemia is usually asymptomatic but may present as a (severe) bleeding tendency [20]. It inherits either autosomal recessively or dominantly. Thrombosis is a reported complication of dysfibrinogenemia in 10 to 30% of patients and there may be impaired wound

healing [20]. Recurrent miscarriage is seen in both afibrinogenemia and dysfibrinogenemia [20].

Hypofibrinogenemia, which is inherited in an autosomal dominant pattern, is defined as fibrinogen <500mg/L. It also presents with a bleeding tendency. In an Iranian series of 60 patients, all with a fibrinogen level below 1 g/L, 75% reported cord bleeding, 70% oral cavity or nose bleeding, 50% joint bleeding or uterus bleeding, 40% postoperative- or post-partum bleeding, and 5% central nervous system bleeding [23].

FII deficiency

FII deficiency is an autosomal recessive disorder. Severe FII deficiency can lead to bleeding of the umbilical cord, joint, muscle and mucosal bleeding, central nervous system bleeding and bleeding after invasive procedures. In a case series of 13 patients 15% of patients had had an umbilical cord bleeding and 15% a central nervous system bleeding [24]. In 39 severe cases reported in the literature intracerebral and gastrointestinal bleeding was seen in 12% of patients, and haemarthrosis in 42%[25]. Spontaneous haematomas/bruising were seen in 60% of patients, bleeding after tooth extractions in 36%, and 20% of females reported menorrhagia.

FV deficiency

FV deficiency is an autosomal recessive disorder. The bleeding tendency in FV deficiency is variable and does not correlate well with the level of FV, especially in the low (<5%) range [22]. The explanation for this heterogeneity is unknown, but compensation by platelet FV may play a role [26]. According to Iranian and North American registries the most common symptoms associated with FV deficiency were bleeding from mucous membranes (e.g. epistaxis, menorrhagia in females) and post-traumatic bleeding following surgery or delivery, which occurred in approximately half of patients[27]. Haemarthroses and muscle haematomas were present in around one quarter of patients, and severe bleeding manifestations (e.g. intracranial or gastro-intestinal haemorrhages) were rare and confined to patients with undetectable FV levels. Despite this rather benign clinical picture there have been several reports in the literature on patients presenting with life-threatening neonatal or perinatal intracranial haematoma's [27].

FV & FVIII deficiency

Combined FV & FVIII deficiency is an autosomal recessive disorder. Individuals with a FV and FVIII deficiency do not have a more severe bleeding tendency than individuals with either a FV or a FVIII deficiency [28]. Those affected usually have a mild to moderate bleeding tendency, with mucous membrane bleeding like epistaxis (19 to 77%), gum bleeding (49 to 64%), menorrhagia (66 to 100%) and post-traumatic/post-surgery (62 to 85%) as the most common manifestations [29]. Joint and muscle bleedings were reported in 25% and 7% of 27 patients in one series, but it was not reported whether these bleedings were spontaneous [23]. In the same patient series 22% reported cord bleeding and 4% central nervous system bleeding.

FVII deficiency

FVII deficiency is an autosomal recessive disorder. The clinical manifestation is heterogeneous. There is a weak correlation between the level of FVII and bleeding tendency. Mild disease will lead to easy bruising, bleeding from mucous membranes or menorrhagia [5]. Severe and homozygous FVII deficiency may lead to haemarthrosis, cerebral haemorrhage and catastrophic haemorrhage after parturition, surgery or injury [5, 30]. In a large series of 73 homozygous individuals intracranial haemorrhage (2%), gastrointestinal bleeding (17%), haemarthrosis (13%), epistaxis (58%), gum bleeding (38%), easy bruising (37%), haematoma (15%), hematuria (10%) and menorrhagia (73%) were described [31]. Interestingly, 19% of heterozygous individuals reported spontaneous bleeding, though severe spontaneous haemorrhages (intracranial and gastrointestinal) were not reported in this series [31]. In contrast, in a series of 49 patients with <10% FVII 17% reported central nervous system bleeding and 14% gastrointestinal bleeding [23].

FX deficiency

FX deficiency is an autosomal recessive disorder. The risk of bleeding correlates well with coagulation factor activity level [19]. Severe bleeding occurs in patients with factor levels below 10%, and includes umbilical cord, intracranial, joint and muscle bleeding and excessive bleeding after surgery or trauma. Heterozygous individuals can have a bleeding tendency. [32].

FXI deficiency

FXI deficiency can be inherited autosomal recessive and autosomal dominant[33]. It is the mildest of the rare bleeding disorders, with bruising, epistaxis and menorrhagia as the most common manifestations [5, 14]. The correlation between coagulation factor activity and clinical bleeding is poor [19]. This may (in part) be explained by the coexistence of other coagulation disorders. In some patients with severe FXI deficiency a combined FXI and von Willebrand disease has been reported, leading to a more severe bleeding tendency [16]. Excessive bleeding has also been reported in heterozygous individuals. A single study from the United Kingdom showed that 48% of heterozygous individuals had a bleeding tendency, [34]. Comparison of histories between partially deficient and non-deficient individuals demonstrated a higher prevalence of menstrual problems (41% vs. 18%), an increase in significant bruising (38% vs. 14%), an increased likelihood of excessive bleeding after dental extractions (51% vs. 9%), and an increased number of blood transfusions after interventions (56% vs. 0%). Bleeding in heterozygous individuals has also been described to be related to genetically compound disease, other factor deficiencies or defects, or platelet dysfunction in some cases [12-18].

FXIII deficiency

FXIII deficiency is an autosomal recessive disorder. This deficiency has serious clinical consequences. Of 112 cases reported in the literature from 1978 23 individuals (21%) had died of bleeding, and notably 22 of these were relatives of established cases, for whom the diagnosis was highly likely but could not be proven [35]. Umbilical cord bleeding was seen in

73-80% of cases and may be delayed up to 19 days after birth; surgical bleeding in 84%; superficial bruising in 58-60%; subcutaneous haematomas in 55-58%; intracranial bleeding in 25-30%; mouth and gum bleeding 30-48%; muscle bleeding 27%; and joint bleeding in 24-55% of reported cases [35, 36]. Habitual abortions (50% of women) and menorrhagia (35% of women) are also frequent. Women with a complete deficiency are unable to carry a pregnancy to term [36, 37]. Impaired wound healing is another symptom patients may experience [37]. It is currently not clear if people with a heterozygous factor XIII deficiency have an increased bleeding risk[38].

Alpha-2-antiplasmin deficiency

Alpha-2-antiplasmin deficiency is an autosomal recessive disorder. Most patients with alpha-2-antiplasmin deficiency experience severe bleeding appearing during childhood. Umbilical cord bleeding has been described, as well as intramedullary haemorrhage into the diaphyses of long bones. Heterozygous individuals can be asymptomatic or have milder symptoms, mostly occurring after trauma, surgery or dental extractions. With age the plasma levels decrease which can lead to increase in symptoms [6, 7].

Plasminogen activator inhibitor deficiency

PAI-1 deficiency is an autosomal recessive disorder. The bleeding symptoms in PAI-1 deficiency are usually mild to moderate. Bleeding after surgery is a common complication as well as abnormal bleeding after dental procedures. Most bleeding episodes are post-traumatic. Epistaxis has also been described as well as menorrhagia, sometimes leading to iron deficiency anaemia or even the need for transfusion [8, 9].

1.3. Health-related quality of life

Health-related quality of life has not been evaluated in patients with rare bleeding disorders.

1.4. Laboratory

The laboratory diagnosis of rare bleeding disorders consists of screening assays, confirmation assays, and global assays. Though characterization of the bleeding disorder is possible through this diagnostic cascade, either or not in combination with genotypic confirmation, there is no test available that sufficiently predicts bleeding phenotype. In addition, all currently available laboratory tests necessitate venepuncture. Non-invasive and/or home-based assays do not exist as yet.

Screening assays

If a bleeding disorder is suspected, clinicians will request coagulation tests and tests to exclude disorders that influence coagulation, such as thyroid disease or liver disease. A routine screening assay generally includes the following tests: activated partial thromboplastin time (APTT), prothrombin time (PT) and fibrinogen activity or thrombin time (TT); platelet function analyzer (PFA), von Willebrand factor activity and antigen, FVIII,

fibrinogen, platelet count, haemoglobin, leucocytes & differentiation, peripheral blood smear, creatinine, ALAT (alanineaminotransferase) and TSH (thyroid stimulating hormone). A prolonged aPTT or PT without another deviating test may indicate a rare bleeding disorder, if acquired diseases and anticoagulant use have been ruled out. A prolonged PT indicates a deficiency of FVII, FX, FV, FII, or fibrinogen. A prolonged aPTT indicates a deficiency of either prekallikrein, fibrinogen, FII, FV, FVIII, FIX, FXI of FXII. A prolonged aPTT and PT suggests that the abnormality is due to a deficiency in FX, FV, FII or fibrinogen.

Confirmation assays

Specific factor assays for each different coagulation factor confirm the specific rare bleeding disorder, and are necessary to give a rough indication of the level of deficiency (severe, moderate, mild). Functional assays are indicated if antigen levels are normal, slightly reduced or increased because they can confirm qualitative factor disorders. Genetic testing is not routine practice, as it does not have direct clinical implications, but may be indicated in case of preconception counselling.

Global assays

The Nijmegen Haemostasis Assay (NHA) measures thrombin and plasmin generation in a single well and allows the detection of coagulation, fibrinolysis and their interplay in a single assay [39]. Preliminary data of 41 patients suggest that the NHA can distinguish fibrinogen disorders and FII, FV, FVII, FX and FXIII deficiencies [40].

1.5. Genetics

In general, mutations in genes that encode for the corresponding coagulation factor are the cause of the bleeding disorder, and mutations are almost always unique to single kindred (Table 2~~Table 2~~). The combined deficiency of FV & FVIII is caused by mutations in genes encoding for proteins involved in intracellular transport [41]. Deficiencies in the vitamin K dependent coagulation proteins (FII, FVII, FIX and FX) can be caused by mutations in genes encoding enzymes involved in post-translational modification and in vitamin K metabolism [42].

In approximately 10% to 20% of patients, no putative mutation is found with conventional genetic techniques that scan the ‘usual suspect’ genes. Cases were no putative mutation has been found may be due to defects in non-coding regions or in genes coding for regulators of intracellular transport and posttranslational modifications of coagulation factors.

Table 2 General genetic features of the rare bleeding disorders [10, 23]

Disorder factor	Gene on chromosome
Fibrinogen	4
II	11
V	1
V & VIII	18

VII	13
X	13
XI	4
XIII	A subunit: 6 B subunit: 1
PAI-1	7
Alpha-2-antiplasmin	17

1.6. Management

Many patients and bleeding episodes will not require therapy: in a North-American registry study in 294 patients with a rare bleeding disorder half of all bleeding episodes did not require therapy [11]. On the other side of the treatment spectrum are patients with severe FX and FXIII deficiency, who may require lifelong prophylaxis treatment. If treatment is needed, the optimal treatment is often unknown as high-quality evidence is lacking. Both factor replacement therapy and non-transfusional treatment are available at present. The backbone of treatment is factor replacement, either in the form of recombinant factor, factor concentrate, prothrombin complex concentrate or plasma products (in order of preference and depending on availability). Non-transfusional treatment includes antifibrinolytic agents (tranexamic acid or ε-aminocaproic acid), which can be used as a supplement to factor replacement in major bleedings, and instead of replacement therapy in minor bleeding. Treatment-related complications have been reported and include viral seroconversion, anaemia, allergic reactions and venous access device-related events [11]. Cases of inhibitors to FV and FXI have been reported.

1.7. Ageing and coagulation

Aging has a distinct effect on coagulation in healthy individuals, with progressive laboratory evidence of increased coagulation activity as individuals age [43]. The plasma concentrations of fibrinogen, factor VII, factor VIII, von Willebrand factor (VWF), factor IX, factor XII, high molecular-weight kininogen, and prekallikrein increase with progressing age in healthy humans [44]. In centenarians, higher levels of fibrinogen, FII and FX have been found, compared to younger individuals, whereas levels of FVII, α2-antiplasmin and PAI did not differ between age groups [45]. It is unknown whether these changes in factor concentrations occur in patients with RBD and if they influence clinical phenotype. In von Willebrand disease age-dependent laboratory change and bleeding phenotype has been described. Von Willebrand factor and FVIII levels increased with age in type 1 patients, with no mitigation in bleeding phenotype. In contrast, in type 2 patients von Willebrand parameters do not increase with age and ageing in these patients is accompanied by increased bleeding [46].

1.8. Arteriothrombotic events and bleeding disorders

Prospective studies have shown that elevations of fibrinogen, FVII and PAI-1 confer a risk of atherothrombotic disease, including recurrent myocardial infarction in young men in the case

of high PAI-1 [43]. There is little knowledge to whether patients with RBD are protected from arterial thrombosis. An Israeli study indicates that severe factor XI deficiency is probably protective against ischemic stroke, but not against acute myocardial infarction [47]. In von Willebrand disease it has been demonstrated that patients have a lower prevalence of coronary heart disease, acute myocardial infarction and ischemic stroke compared to the general population [48], whereas there is conflicting evidence as to the risk of cardiovascular disease in haemophilia patients [49, 50].

2. RATIONALE

Because of their extreme rarity very few clinical centers have the opportunity to follow up significant numbers of patients; consequently the amount of information available on diagnosis and treatment of rare bleeding disorders is very limited. RBiN will research three novel themes - genotype-phenotype correlations, health-related quality of life and innovative diagnostics - in a patient population that has not been described to date (the Dutch patient population).

Mapping Dutch individuals with rare bleeding disorders for the first time, including health-related quality of life, and age related comorbidities

At present, there is very little information on rare bleeding disorders in the Netherlands, their clinical presentation, bleeding score, bleeding episodes, health-related quality of life, laboratory parameters, genetics and treatment. Therefore, patients with rare bleeding disorders and their doctors have articulated the need for a Dutch study: the Netherlands Haemophilia Society (Nederlandse Vereniging van Hemofilie Patiënten, NVHP) and all treatment centers are co-initiators of this study.

Examining genotype-phenotype correlations

Genotype-phenotype correlations are important in the prognosis and management of patients with rare bleeding disorders, especially disorders in which there is a weak or non-existent association between coagulation factor activity level (laboratory phenotype) and clinical bleeding severity (clinical phenotype), i.e. FV, FVII and FXI deficiencies.

Comparing rare bleeding disorders to haemophilia A and B,

Often, the rare bleeding disorders are categorized as less severe compared to haemophilia A and B. By using similar questionnaires as the upcoming (2017) HiN study (Haemophilia in the Netherlands), a direct comparison of the clinical picture of the distinct rare bleeding disorders haemophilia A and B is possible.

Innovations in diagnostics: non-invasive testing, global assays and whole exome sequencing

Treatment and prophylaxis decisions require frequent venepuncture. Non-invasive testing does not exist yet, but would be extremely beneficial to patients and give way to a future perspective of home-based monitoring combined with home-based treatment. Saliva biomarkers that correlate with anticoagulation status have been identified in coumarin users in Radboud university medical center (unpublished data). RBiN will examine whether these biomarkers correlate with the clinical and laboratory phenotype in patients with rare bleeding disorders and if so, whether we can monitor treatment or rely treatment decisions on these biomarkers.

Precise, personalised treatment of the rare bleeding disorders is hampered by the lack of an adequate test that reflects patients' bleeding potential. At present, treatment decisions are based on clinical phenotype, and standard consensus-based approaches which give the most accurate indication available, but still need fine tuning. Because the Nijmegen

Hemostasis Assay (NHA) measures the key enzymes in both coagulation and fibrinolysis, it might give clinicians a more accurate idea of the haemostatic potential of their patients than currently used tests can. Ideally, this would enable tailored prophylaxis and ad hoc treatment change.

The diagnosis of a rare bleeding disorder is based upon a step-wise conventional laboratory approach as described above (1.4 Laboratory). This strategy is time consuming and expensive, due to the high number of laboratory assays that may be needed (sequentially) to establish a diagnosis. In addition, repeat testing is mandatory for many assays because they are highly variable and dependent on preanalytical and analytical factors. On the other hand, the cost of advanced molecular diagnosis such as whole exome sequencing (WES) is falling, and its practice is ever more common and developed. In addition, WES can trace more genetic abnormalities than current genetic analysis using gene panels can, thereby possibly explaining the differences in bleeding tendency in patients with rare bleeding disorders.

3. OBJECTIVES

Our primary objectives are to:

- Describe the epidemiology, clinical presentation, bleeding score, bleeding episodes, quality of life, laboratory parameters, genetics and treatment of *homozygous* and known *heterozygous* individuals (of all ages) with rare bleeding disorders (disorders of fibrinogen, FII, FV, FV & VIII, FVII, FX, FXI, FXIII, alpha-2-antiplasmin and PAI-1 deficiency) in the Netherlands;
- Examine the relationship between the clinical and laboratory presentation (clinical and laboratory phenotype), and between phenotypes and genetics (genotype);
- Examine the relationship between quality of life, phenotype and genotype;
- ~~Validate the established factor activity levels for patients to remain without symptoms.~~

Our secondary objectives are to:

- Validate the established factor activity levels for patients to remain without symptoms.
- Compare the clinical presentation, bleeding score, quality of life and laboratory parameters of individuals with a rare bleeding disorder (disorders of fibrinogen, FII, FV, FV & VIII, FVII, FX, FXI, FXIII, alpha-2-antiplasmin and PAI-1 deficiency) to those of individuals with haemophilia A or B in cooperation with the HIN-6 investigators
- Establish a firm base for a future Dutch registry for *homozygous* and known *heterozygous* individuals with rare bleeding disorders
    - To develop a standard set of patient-reported, clinical and administrative data to be collected on a regular basis
- Liaise with the pro-RBDD study, a similar study in Italy, to work towards a pan-European study linking phenotype to genotype in individuals with rare bleeding disorders
- To assess if the NHA can distinguish mild clinical phenotypes in patients with similar factor activity levels
- To evaluate the usefulness of saliva coagulation biomarker tests in the management of patients with a rare bleeding disorder
- To examine whether age-dependent laboratory changes in factor concentrations and fibrinolysis occur in individuals with rare bleeding disorders and if so, whether they influence clinical phenotype
- To evaluate if patients with rare bleeding disorders are protected from arterial thrombosis

4. STUDY DESIGN

RBiN is a cross-sectional observational study. All participants will be asked to fill out questionnaires, will be interviewed, and asked to provide blood and saliva samples. The blood samples will be used for analyses and to set up a biobank.

5. STUDY POPULATION

5.1. Population (base)

Based on the prevalence in the general population of 1:500,000 to 1 per 2 million persons [1, 2], and a total population of 17 million, there should be 129 to 145 individuals with a homozygous rare bleeding disorder in the Netherlands. This could be higher taking into account the possible influences of high-risk populations such as Ashkenazi or Iraqi Jews with high FXI deficiency rates. To date, 337 patients with a rare bleeding disorder are known to the eight Dutch centers that treat such patients. Approximately one third of these patients are homozygous; and around two thirds are heterozygous individuals. [1]

All known homozygous and heterozygous patients with a rare bleeding disorder will be invited to participate.

Heterozygous prevalence in the Netherlands have not been evaluated, but can be calculated according to the Hardy-Weinberg principle[2] to be 93,000 to 99,000 persons, or 0.55% to 0.58% of the Dutch population (5.5 to 5.8 per 1,000 persons) for all rare bleeding disorders combined. Tough the estimated number of heterozygous persons in the Netherlands is much higher than the number of persons known to one of the eight treating centers (~179 heterozygous patients[3]), we expect that the individuals with a known heterozygosity represent a subset with a more severe clinical presentation, who are therefore of interest to our research question.

Characteristics of the Dutch source population

The characteristics of the Dutch population (both homozygous and heterozygous) are largely unknown and are a part of this study. Numbers of known patients per rare bleeding disorder are presented in

Table 3

~~Table 3~~.

[1] A quick inventory in the Radboud university medical center learned that two thirds of patients were heterozygous individuals whereas one third were homozygous individuals. Based on an inventory of 63 medical files performed on July 8, 2016: 65% missing information, 24% heterozygosity, 11% homozygosity

[2] The Hardy-Weinberg proportion stipulates that for a single locus with two alleles denoted 'A' and 'a' with population frequencies p and q respectively, the expected genotype frequencies are p^2 for the AA homozygous individuals, q^2 for the aa homozygous individuals, and 2pq for the heterozygous individuals. The sum of these frequencies must be 1, thus p2 + 2pq + q2 = 1 and p + q=1.

Table 3 Prevalence of rare bleeding disorders in the Netherlands

Deficiency	Estimated prevalence homozygosity in general population as described in the literature [1, 2]	Estimated prevalence homozygosity in the Dutch general population	Patients known at present in the Netherlands (homozygous/heterozygous/unknown)
Fibrinogen	1:1 million	17	28
II	1:2 million	9	9
V	1:1 million	17	22
V & VIII	1:1-2 million	9-17	4
VII	1:500,000	34	92
X	1:1 million	17	10
XI	1:1 million	17	88
XIII	1:1-2 million	9-17	16
PAI-1	Unknown	Unknown	39
Alpha-2-antiplasmine	Unknown	Unknown	29
	Total	*129-145*	*337*

Abbreviations: CI: confidence interval

$ Based on a population figure of 17,024,133 (www.cbs.nl/nl-nl/visualisaties/bevolkingsteller)

* Based on a quick inventory of participating sites, which are all sites in the Netherlands licensed to treat rare bleeding disorders. Homozygosity or heterozygosity largely unknown

Willingness of patients to participate

We expect that a high proportion of patients with rare bleeding disorders are willing to participate in RBiN. Patients are aware of the rarity of their disorder and the lack of research. The Netherlands Hemophilia Society (Nederlandse Vereniging van Hemofilie Patiënten, NVHP) is a co-initiator of RBiN and a patient representative takes seat in the steering committee. Based on the response rate of the HiN studies we expect a (similar) response rate of 70% [51].

5.2. Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Established homozygous or known heterozygous rare bleeding disorder due to deficiency or dysfunction of fibrin, FII, FV, FV & FVIII, FVII, FX, FXI, FXIII, alpha-2-antiplasmin and PAI-1 ;
- Age 1 year and older;

- For patients ≥ 16 years old; written informed consent.
- For patients 12-16 years old; written informed consent from both the patient and their parents/legal guardian(s).
- For patients <12 years old; written informed consent from their parents/legal guardian(s).

5.3. Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- No informed consent given;
- Residency outside of the Netherlands

6. METHODS

6.1. Study procedures

Patients will be approached by their own treating physician. Data will be collected through questionnaires, a clinical interview, a blood and saliva sample obtained from each participant, and thorough review of electronic patient records. An overview of study procedures is given in Table 4. All procedures are study related. In case a physician visit with the treating physician is already planned, or in case a venepuncture for regular diagnostics or treatment is already planned, this can be combined with the study procedures to prevent an extra visit.

Table 4 Overview of study procedures for each adult subject

Procedure	Outcome
Study physician visit	
• Clinical interview	• Bleeding score (ISTH-BAT) • RBD bleeding score[52] • Children: iCHEC
Questionnaires	
• Self-administered	• Demographic characteristics (generic) • Socio-economic characteristics (generic) • Clinical characteristics (bleeds, treatment), HIV status (patients born before 1985) and hepatitis C status (patients born before 1992) • Medical history (generic) • Sexuality (generic) • General health status (Rand-36/Cho-KLAT) • Sports and physical activity (MAQ/HeptestQ) • Adherence (VERITAS-pro) • Pain (generic) • Needle phobia (generic) • Experience with care (generic) •
Electronic patient records	
	• Date of birth • Sex • Baseline factor activity level • Baseline factor antigen level • Treatment type (prophylactic treatment or on-demand treatment) • Body weight and height • Bleeding score at diagnosis • Relevant previous medical history, i.e. arterial thrombosis, atherosclerosis, medication use, pregnancies and outcomes
Blood samples: 72-83 ml	
• Haemostasis	• Screening tests - PFA - Haemoglobin

		- Thrombocytes - VWF ristocetin cofactor - VWF antigen - VWF collagen binding assay - FVIII act one stage - FVIII chromogen - aPTT - PT - Fibrinogen - Thrombin time	- Leucocytes & differentiation - Peripheral blood smear - Creatinine - ALAT - TSH - D-dimer - Antitrombin - Protein C - Protein S - TFPI
	• Diagnostic tests	- FII - FV - FVII - FIX	- FX - FXI - FXII - FXIII
	• Global assay	- Nijmegen Haemostasis Assay	
• Fibrinolysis	• PAI-1 • TPA • Plasminogen	• α2 –antiplasmin • TAFI	
• Other	• DNA (whole exome sequencing) • Inhibitor assays (only in people ever treated with clotting factors) • Lupus anticoagulans • Anti β2 glycoprotein I		
• Biobank	• 2 x 10 ml citrate		
Saliva sample			
• Coagulation	• Biomarkers of coagulation		

Abbreviations: ISTH-BAT: International Society on Thrombosis and Haemostasis/Scientific and Standardization Committee Bleeding Assessment Tool

6.2. Data collection

Questionnaires

Patients will receive a personal log on code to an electronic questionnaire after informed consent. They will be reminded after one month to fill out the questionnaire online. Patients unwilling or unable to fill out the questionnaire electronically will be provided with a hard copy of the questionnaire. The questionnaires can be found in separate documents. Children's versions of the questionnaire will be available for children up to 17 years old. Whenever possible, questionnaires specifically validated and designed to be filled out by children will be used. In general, children from 12-17 years old will be able to fill out the questionnaire independently. For children under 12 years old, parents will fill out most of the questions.

The complete questionnaire will take approximately 40-120 minutes to complete, depending on which questions are relevant to the patient. For example, the haemophilia activity list of approximately 50 questions will only be filled out by patients with chronic joint impairment, a

minority in this patient group. The VERITAS-PRO questionnaire will only be performed in patients who are on prophylaxis and patients born after 1992 will not fill out questions about hepatitis C and HIV infections. There are also parts of the questionnaire only for women or certain age-groups. Therefore, we expect that close to no patients will have to fill out all the questions. The questionnaire can be paused at any time and continued later on.

The patients will submit the questionnaires digitally. The questionnaires will be checked and discussed briefly to verify during the patient visit. The bleeding scores are entered directly into the eCRF.

Blood sampling

All participants will be asked to provide a blood sample during a visit to the haemophilia treatment center. Sampling of 1-5% of an individual's total blood volume is deemed safe according to a systematic review of the literature and of existing guidelines[53]. For this study we will set the maximum around 2.5% of the total blood volume, or 2mL/kg based on a total circulating blood volume of 80mL/kg in persons weighing 2kg or more.

In adults and children ≥12 years old, roughly 67 ml blood will be drawn through a study-related venepuncture. Forty-seven ml of this sample will be used for this study while the remaining 20 ml will stored in the Radboud university medical center Biobank for future research. Additional information about the Biobank is provided in separate documents.

Blood samples will be used for the experiments specified in table 4.

In children under 16 years of age, whole exome sequencing will not be performed. Also, the following tests are not performed under 12 years of age: ALAT, TSH, creatinine, lupus anticoagulans and d-dimer. Therefore, only 47mL of blood will be needed for these children. For children under 8 years of age, in addition there will be no blood drawn for the biobank so that they only need to supply 27mL of blood.

Remaining material can be used for future research. In this case, studies will be performed according to the protocol Biobank Hematologie HEMBB (IRB Registration number: CMO Arnhem-Nijmegen 2013/064). In case the research would not be within the scope of HEMBB, the CMO will be consulted in advance.

Saliva sampling

Whole saliva will be collected in the morning when patients did not drink more than 2 glasses of alcohol on the evening before and did not eat 1 hour before collection. In case of infections

in the oral cave or a severe cold, patients will be excluded. Before saliva collection the mouth will be rinsed with water and donors will be allowed to chew on 25 cm2 sheet of parafilm (Parafilm® M) for 1 minute. Produced saliva will be collected into a 50 ml tube and centrifuged at 10,000g for 10 minutes. Samples will be divided in aliquots of 250 µl and stored at -80°C. Stimulated whole saliva collection will be used because of its non-invasive approach instead of *e.g*. glandular saliva donation, and since stimulated whole saliva has been found to have a large portion of peptides and proteins derived from the parotid glands. Specimens will be proceeded with several techniques including ELISA and proteomics.

Saliva samples are only collected in patients ≥4 years old.

Electronic patient records

The clinical data will be collected from the medical charts by the research team using a case report form. Each patient will be assigned a unique study code, as described XXXX. A copy of the case report form will be sent to the coordinating center (Radboud university medical center) for data entry and a copy will be kept at the original center.

Whole exome sequencing

In all patients over 12 years of age, whole exome sequencing will be performed. A filter will be used to get the results of 135 bleeding related OMIM-proved genes involved in haemostasis.

6.3. Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

6.4. Replacement of individual subjects after withdrawal

Not applicable as all known patients will be invited to participate.

6.5. Follow-up of subjects withdrawn from treatment

Not applicable as this study has no follow-up.

6.6. Premature termination of the study

If the recruitment of subjects goes unexpectedly too slow within a reasonably time period the study ends prematurely. Too slow a recruitment is defined as less than one fifth of known patients, thus, less than 60 patients, within one year of the start of the study.

7. STATISTICAL ANALYSIS

Statistical analysis will be performed by the research team at Radboud university medical center. First we describe the general outline of statistical analyses, then we discuss specific statistical procedures for the primary and secondary objectives separately, if appropriate.

In general, continuous variables will be described using standard summary statistics (e.g. mean, medians and corresponding 95% confidence interval (CI),); categorical variables will be described using frequencies and percentages with their 95% CIs. Group differences will be determined using analysis of variance (ANOVA) or Student's t-test for continuous variables and Fisher's exact test for categorical variables (Fisher-Freeman-Halton exact test for variables with more than two categories). Non-parametric tests will be used for results with non-normal distributions. Two-sided p-values smaller than 0.05 will be considered statistically significant. Because of the explorative character of this study no correction for multiple testing will be applied.

The following parameters will be categorized when appropriate:

- Bleeding score (ISTH-BAT): ≥5 abnormal for women, ≥3 abnormal for men
- Clinical bleeding severity [19]:
    - Asymptomatic: no documented bleeding episodes
    - Grade I bleeding: bleeding that occurred after trauma or drug ingestion (antiplatelet or anticoagulation therapy)
    - Grade II bleeding: spontaneous minor bleeding (bruising, ecchymoses, minor wounds, oral cavity bleeding, epistaxis and menorrhagia)
    - Grade III bleeding: spontaneous major bleeding (intramuscular haematomas requiring hospitalizations, haemarthrosis, bleeding of the central nervous system, the umbilical cord or the gastrointestinal tract)

7.1. Primary objectives

- *Describe the epidemiology, clinical presentation, bleeding score, bleeding episodes, quality of life, laboratory parameters, genetics and treatment of homozygous and known heterozygous individuals (of all ages) with rare bleeding disorders (disorders of fibrinogen, FII, FV, FV & VIII, FVII, FX, FXI, FXIII and alpha-2-antiplasmin and PAI-1 deficiency) in the Netherlands;*

  We will use descriptive statistics using means, SD and medians. Interquartile ranges will be used when appropriate. Prevalence will be reported with 95% confidence intervals.
- *Examine the relationship between clinical and laboratory presentation (clinical and laboratory phenotype), and between phenotypes and genetics (genotype);*

  Descriptive statistics will be used. Regression analysis will be used to determine the association between coagulation factor activity level (dependent variable) and clinical

bleeding severity as a continuous variable (independent variable), with adjustment for age at data collection, sex, etcetera.

- *Examine the relationship between quality of life, phenotype and genotype;*
  Descriptive statistics will be used. Regression analysis will be used to determine the association between quality of life (dependent variable) and clinical bleeding severity or coagulation factor activity level as a continuous variable (independent variable), with adjustment for age at data collection, sex, etcetera.
- *Validate the established factor activity levels for patients to remain without symptoms.*
  Descriptive statistics will be used.

7.2. Secondary objectives

- *Compare the clinical presentation, bleeding score, quality of life and laboratory parameters of individuals with a rare bleeding disorder (disorders of fibrinogen, FII, FV, FV & VIII, FVII, FX, FXI, FXIII and alpha-2-antiplasmin and PAI-1 deficiency) to those of individuals with haemophilia A or B (HiN study data will be shared)*
  Descriptive statistics will be used. Group differences will be determined using the statistics as described in Statistical Analysis above.
- *Establish a firm base for a future Dutch registry for homozygous and known heterozygous individuals with rare bleeding disorders (disorders of fibrinogen, FII, FV, FV & FVIII, FVII, FX, FXI, d FXIII and alpha-2-antiplasmin and PAI-1 deficiency):*
  - *To develop a standard set of patient-reported, clinical and administrative data to be collected on a regular basis*

  No statistics are involved in this objective.
- *Liaise with the pro-RBDD study, a similar study in Italy, to work towards a pan-European study linking phenotype to genotype in individuals with rare bleeding disorders*
  No statistics are involved in this objective.
- *To assess if the NHA can distinguish mild clinical phenotypes in patients with similar factor activity levels*
  We will calculate diagnostic accuracy comparing NHA to ISTH BAT.
- *To evaluate the usefulness of saliva coagulation biomarker tests in the management of patients with a rare bleeding disorder*
  Descriptive statistics will be used. Regression analysis will be used to determine the association between saliva biomarker tests (dependent variable) and coagulation factor activity level as a continuous variable (independent variable), with adjustment for age at data collection, sex, etcetera.

- *To evaluate the use of WES in explaining differences in bleeding tendency.*
  Descriptive statistics will be used.
- *To examine whether age-dependent laboratory changes in factor concentrations and fibrinolysis occur individuals with rare bleeding disorders? And if so, do they influence clinical phenotype?*
  Descriptive statistics will be used. Regression analysis will be used to determine the association between age (dependent variable) and coagulation factor activity level as a continuous variable (independent variable), with adjustment for age at data collection, sex, etcetera.
- *To evaluate if patients with rare bleeding disorders are protected from arterial thrombosis*
  Descriptive statistics for continuous variables will be presented as medians and 25–75% interquartile ranges (IQR), because data are not normally distributed. For comparison of proportions, the chi-squared test or the Fisher's exact test will be used. Wilcoxon tests will be performed for comparison of age and coagulation factor levels between groups. Standardized morbidity ratios (SMR) will be calculated to estimate the rate of overall arterial thrombosis and of coronary heart disease, acute myocardial infarction and ischemic stroke separately in patients relative to that of the reference population adjusted for age and sex.

8. ETHICAL CONSIDERATIONS

8.1. Regulation statement

This study will be conducted in accordance to the principles of the Declaration of Helsinki, ICH guidelines on Good Clinical Practice (GCP) and in accordance with the Medical Research Involving Human Subjects Act (WMO). The local investigator is responsible for ensuring that the study will be conducted in accordance with the protocol, ethical principles, ICH-GCP guidelines and the WMO.

8.2. Recruitment and consent

Subjects will be informed about the study by their treating physician. A minimum of one week will be allowed to consider their decision to participate after giving informed consent. The patient information letter and informed consent (in Dutch) are attached as separate documents.

8.3. Objection by minors or incapacitated subjects

For minors the “Code of conduct relating to expressions of objection by minors participating in medical research” (“Gedragscode verzet”) is applicable. This code of conduct was approved by the Board of the Netherlands Association for Paediatric Medicine (NVK) on 21 May 2001 and published in NVK Newsletter no. 3, June 2001.

8.4. Benefits and risks assessment, group relatedness

There is a possible minor bleeding risk for people participating in this study connected to collection of blood. However, this is a very minor risk considering the experience of patients and nurses. Blood of patients having RBDs is routinely tested during regular visits to treatment centers and risks associated to blood sampling are considered to be negligible.

Patients will not directly benefit from participating in this study but in the long term this study contributes to our knowledge of the rare bleeding disorders and will increase awareness of these disorders amongst health care workers, as such improving diagnostics and care.

Since the population of patients having RBDs is very small, it is important to include all patients, also minors (children <18 years), in the study (around one third of known patients are minors). Therefore this study may be regarded as group-related.

8.5. Compensation for injury

The sponsor/investigator has liability insurance in accordance with article 7 of the WMO.

The risk associated with the study investigations is considered negligible; therefore no additional insurance to compensate for injury (in Dutch: proefpersonenverzekering) is necessary and dispensation is requested.

8.6. Incentives

Subjects will not receive any financial compensation or treatment through participation in this study. After participation, patients will receive a small present with the study logo, e.g. a keychain. Participants will also receive a report of the most important study results.

9. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

9.1. Handling and storage of data and documents

The handling of personal data complies with the Dutch Personal Data Protection Act (in Dutch: De Wet Bescherming Persoonsgegevens, Wbp). Patient data will be coded by patient study number to protect the privacy of the subjects. Subject identification codes will consist of a research center code and serial number. Subject identification codes will be ascribed on inclusion and these codes will be used throughout the study. The code will not contain any personal identifiers such as date of birth of initials. The key to the identification code will be safeguarded by the participating centers for their own patients.

Data will be stored on protected files on servers according to Standard Operating Procedures of the Department of Haematology. Research data will be kept until publication of the study results and stored for 15 years. Individual patients will not be identifiable within publications or presentations.

9.2. Monitoring and Quality Assurance

According to the NFU guidelines this is a study with negligible risk [54]. Therefore there will be low-intensity monitoring.

9.3. Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

Non-substantial amendments (e.g. typing errors and administrative changes like changes in names, telephone numbers and other contact details of involved persons mentioned in the submitted study documentation) will not be notified to the accredited METC, but will be recorded and filed by the sponsor.

9.4. Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

9.5. Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient’s last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

9.6. Public disclosure and publication policy

The study results will be disclosed unreservedly, and we aim to report the results in a peer-reviewed scientific journal. After approval of the study protocol by the METC this study will be registered in a public trial registry.

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Appendix 1: Patient information letter and informed consent form