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ORIGINAL ARTICLE

Targeted exome analysis in patients with rare bleeding disorders: data from the Rare Bleeding Disorders in the Netherlands study

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Abstract

Background: Rare coagulation factor deficiencies and disorders of fibrinolysis (defined as rare bleeding disorders [RBDs]) present with a heterogeneous bleeding phenotype, and bleeding severity is difficult to predict.

Objectives: Describe underlying rare genetic variants in the Dutch RBD population and investigate the relationship between genotype, laboratory phenotype, and clinical phenotype.

Methods: The Rare Bleeding Disorders in the Netherlands is a cross-sectional, nationwide study conducted between October 1, 2017, and November 30, 2019. Bleeding scores and blood samples were collected during a single study visit. Coagulation factor levels were measured centrally, and targeted exome analysis was performed on 156 genes involved in

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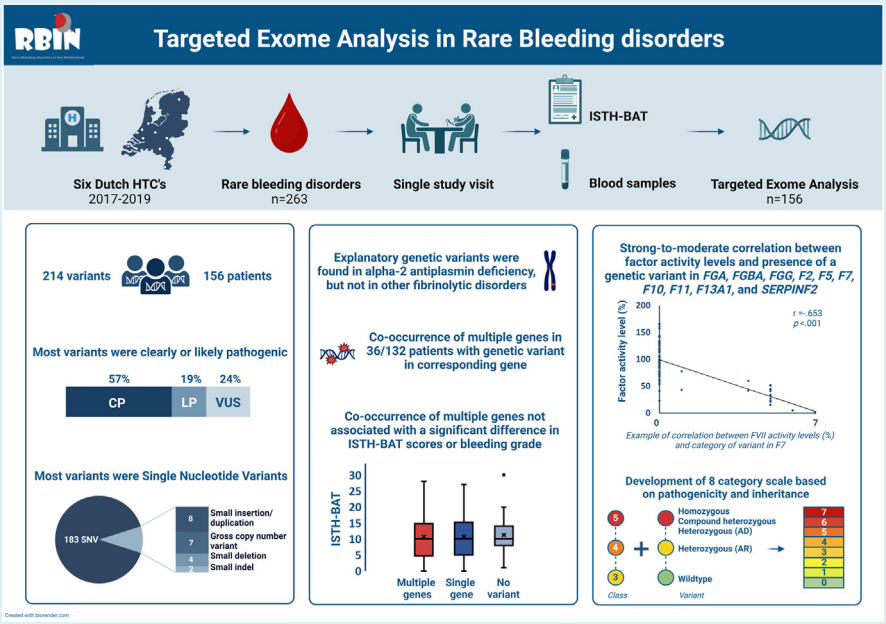
thrombosis and hemostasis. Pathogenicity was assigned according to the Association for Clinical Genetic Science guidelines.

Results: Rare genetic variants specific to the diagnosed RBD were found in 132 of 156 patients (85%). Of the 214 rare genetic variants identified, 57% (*n* = 123) were clearly pathogenic, 19% (*n* = 40) were likely pathogenic, and 24% (*n* = 51) were variants of unknown significance. No explanatory genetic variants were found in patients with plasminogen activator inhibitor type 1 deficiency or hyperfibrinolysis. A correlation existed between factor activity levels and the presence of a genetic variant in the corresponding gene in patients with rare coagulation factor deficiencies and alpha-2-antiplasmin deficiency. Co-occurrence of multiple genetic variants was present in a quarter of patients, but effect on phenotype remains unclear.

Conclusion: Targeted exome analysis may offer advantages over single-gene analysis, emphasized by a number of combined deficiencies in this study. Further studies are required to determine the role of co-occurring hemostasis gene variants on the bleeding phenotype in RBDs.



KEYWORDS
blood coagulation disorders, exome sequencing, fibrinolysis, hemostasis, inherited

Essentials

- In general, rare coagulation factor deficiencies are confirmed by a molecular diagnosis.
- An 8-tier genetic variant system significantly correlated with factor activity levels.
- No variants were found in the SERPINE1 gene in patients with plasminogen activator inhibitor type 1 deficiency.
- Co-occurrence of multiple genetic variants was frequent, with an unknown effect on phenotype.

1 | INTRODUCTION

Rare bleeding disorders (RBDs) encompass a heterogeneous group of rare, hereditary coagulation factor deficiencies, platelet disorders, disorders of fibrinolysis, and bleeding of unknown cause. The Rare Bleeding Disorders in the Netherlands (RBiN) study employed a previously commonly used definition to investigate RBDs. In this study, RBDs are defined as deficiencies of fibrinogen, prothrombin (factor [F]

II), FV, FVII, FX, FXI, FXIII, combined deficiencies of FV and FVIII, as well as FV Amsterdam and the fibrinolytic disorders plasminogen activator inhibitor type 1 (PAI-1), alpha-2-antiplasmin (A2AP) deficiency, and hyperfibrinolysis [1–4]. These RBDs roughly represent 9% of inherited bleeding disorders [5–7].

RBDs are considered monogenic, and most display an autosomal recessive inheritance pattern [8]. Therefore, the prevalence of RBDs is notably higher in regions with a high degree of consanguinity [9,10]. Dysfibrinogenemia and FV Amsterdam, however, follow an autosomal dominant inheritance pattern, similar to some cases of FXI deficiency [11–13]. Heterozygous variants in recessive RBD genes may cause mild factor deficiency. Along the mode of inheritance, penetrance is an important factor that determines genotype-phenotype correlation in RBDs. However, due to incomplete penetrance and variable expressivity, the clinical phenotype in patients with RBDs is heterogeneous and challenging to predict [14,15]. Moreover, prior research on the correlation between factor activity levels and clinical bleeding severity has yielded ambiguous results [2,16,17].

High throughput sequencing (HTS) using a multigene panel has been incorporated into daily practice as a complementary diagnostic tool for RBDs. Especially in patients with well-defined bleeding disorders, diagnostic sensitivity is high (>90%). However, studies exploring HTS in RBDs often lack clinical data [18–25]. Whole exome sequencing (WES) with a multigene panel offers advantage over targeted gene panel sequencing, allowing for potential expansion of other genes analyzed as information on underlying genetic disorders evolves. Moreover, the variable clinical phenotype in patients with RBDs may be partially explained by the presence of gene-gene interaction caused by oligogenic variants, which go unnoticed in single-gene analysis. Some cases suggest that presence of a prothrombotic variant can be associated with a milder bleeding phenotype in patients homozygous for the FVII Lazio variant [26]. Furthermore, gene panel analysis in the diagnostic work-up in 87 patients with a bleeding tendency identified combined carriership of variants in autosomal recessive genes as a potential explanation in 5 patients [22]. Nevertheless, identifying inherited genetic modifiers requires analysis of extensive pedigrees or a considerable number of unrelated patients, posing a substantial challenge in RBDs [24].

Altogether, these studies suggest that bleeding severity may be explained by an interplay of procoagulant, anticoagulant, and fibrinolytic factors. This prompts further exploration into the possibility of additional genetic defects interfering with the hemostatic balance. To unveil underlying genetic variants in thrombosis and hemostasis genes in patients with an RBD, targeted exome analysis was performed in the multicenter RBiN study [2]. The relationship between genotype, laboratory phenotype, and clinical phenotype is investigated in this substudy.

2 | METHODS

The cross-sectional, nationwide RBiN study recruited participants from all 6 Dutch Hemophilia Treatment Centers (HTCs) between October 1, 2017, and November 30, 2019. RBDs were defined as rare coagulation factor deficiencies, FV Amsterdam, and disorders of fibrinolysis. Patients aged ≥1 year were invited to participate if they had a diagnosis of an RBD based on coagulation factor activity levels below the lower limit of normal or a previously proven homozygous or (compound) heterozygous variant in a gene encoding for an RBD. Therefore, both patients and family members with heterozygous variants in RBD genes were included in this study [27]. Patients with A2AP deficiency were included based on A2AP activity levels below the lower limit of normal (<87%) or presence of a (likely) pathogenic variant in *SERPINF2*. All patients with PAI-1 deficiency and hyperfibrinolysis were included in the HTC Nijmegen-Eindhoven-Maastricht (NEM), as this is the national specialized center for disorders of fibrinolysis. Laboratory tests for diagnosis of PAI-1 deficiency or hyperfibrinolysis are performed in patients with a positive family history of a fibrinolytic disorder, an International Society on Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT) > 10, or in patients with a typical fibrinolytic bleeding pattern. PAI-1 deficiency may be diagnosed if PAI-1 activity levels are below the detection limit and PAI-1 antigen level is below the lower limit of normal (3.4 ng/mL) [2,27]. Hyperfibrinolysis is assessed with the use of euglobulin clot lysis time (ECLT). An ECLT ratio is determined by comparing the time for clot lysis in blood samples collected before and after 10 minutes of venous compression, and ECLT ratios of >5.7 or a baseline ECLT < 116 minutes are indicative of hyperfibrinolysis [27]. Design and patient inclusion details were published previously [2].

2.1 | Patient selection

To limit blood sample volume in young children, patients qualified for genetic analysis if they were aged ≥12 years or if genetic analysis had already occurred during a prestudy visit in their own HTC. Additional informed consent was given separately for targeted exome analysis with a gene panel containing thrombosis and hemostasis genes in accordance with the Declaration of Helsinki. The Medical Research Ethical Commission Oost-Nederland approved this study, and this study was registered at ClinicalTrials.gov as NCT03347591.

2.2 | Clinical and laboratory assessment

During a single study visit, clinical phenotype was assessed with the use of the ISTH-BAT (normal range 0-3 for men, 0-5 for women, and 0-2 for children [28]), and clinical bleeding grades were determined based on the 4 categories of severity from the European Network-RBD study (Supplementary Table S1) [16]. Blood samples were obtained for laboratory testing. The central laboratory of the HTC-NEM analyzed the samples, with the exception of global blood count and platelet function analyzer (PFA) tests, which were performed locally. Moreover, baseline coagulation factor activity levels for patients with missing samples and/or on prophylaxis with factor replacement therapy were extracted from local electronic patient records. Fibrinogen

activity levels were measured using the Clauss method. A 1-stage clotting assay with factor-deficient plasma (Cephascreen/PTT-LA FVIII and FIX, CK-Prest FXI, Neoptimal FII, FV, FVII and FX, STA-R Evolution, Stago) measured factor activity levels of FII, FV, FVII, FVIII, FIX, FX, and FXI. FXIII activity levels were determined using the Berichrom chromogenic ammonia release assay (Siemens). von Willebrand factor (VWF) antigen (VWF:Ag) and ristocetin cofactor activity (VWF:RCo) were measured with an automated assay (HemosIL AcuStar VWF Assay Panel [Instrumentation Laboratory]). A chromogenic assay of antiplasmin (STA-Stachrom Antiplasmin [Stago]) measured A2AP activity. Considering the diurnal variation of PAI-1 antigen and activity levels, baseline levels from morning samples were extracted from patient records from HTC-NEM for patients with a PAI-1 deficiency [27]. Additionally, ECLT ratios were extracted from patient records for patients with hyperfibrinolysis. If fibrinogen levels were below the detection limit (250 mg/L), the level was set to 250 mg/L. As the measurement of FXIII activity levels is unreliable in patients with low (<800 mg/L) or high (>6000 mg/L) fibrinogen levels, FXIII activity levels were not reported in these patients [29].

2.3 | Targeted exome analysis

Targeted exome analysis was performed at the Department of Human Genetics of the Radboudumc in Nijmegen, the Netherlands. Genomic DNA was isolated from whole EDTA blood using an automated procedure. Exonic enrichment and library preparation were performed with the Twist Human Core Exome Kit (Twist Biosciences). DNA samples were sheared with a Covaris R230 Focused-ultrasonicator (Covaris), followed by sequencing on a NovaSeq 6000 and/or HiSeq instrument (Illumina). Alignment to the hg19 (GRCh37) reference genome was performed with Burrows-Wheeler Aligner mapping (version 0.5.9-r16) [30]. Variant calling required a minimum exon coverage of >40×. The Genome Analysis Toolkit (version 3.2.2) was used for variant calling, and variants were annotated using an in-house custom diagnostic annotation workflow [31]. Genes known to be involved in thrombosis and hemostasis were selected by a bioinformatic *in silico* filter (HEMOS panel, 156 genes, version 12.8, Supplementary Table S2). The HEMOS panel also included genes associated with bleeding that are not considered coagulation or platelet regulatory genes, such as genes associated with connective tissue disease. Low-quality variant calls (Genome Analysis Toolkit quality depth < 500) were manually inspected and, if necessary, confirmed by standard Sanger sequencing. The copy number inference from the exome reads method was used for the identification of copy number variants [32]. No segregation analysis was performed.

Variants were independently evaluated by 2 clinical laboratory geneticists, who were not blinded to clinical information. Evaluation for possible pathogenicity was based on prior literature, a local database, population frequencies (Genome Aggregation Database [GnomAD] database, v2.1.1 GRCh37/hg19), functional tests, and multiple prediction tools incorporated in the Alamut software [33]. Variants with population frequencies >1% were considered polymorphisms and were excluded from further evaluation. However, the prothrombotic polymorphisms FV Leiden (NM_000130.5:c.1601G>A) and prothrombin G20210A (NM_000506.5:c.*97G>A) were extracted from the unfiltered variant call format files. A real-time polymerase chain reaction-based method (Xpert HemosIL FII & FV [Instrumentation Laboratory], GeneXpert) was performed in patients with insufficient coverage of NM_00506.5:c.*97.

Pathogenicity was assigned to variants using the practice guidelines of the Association for Clinical Genetic Science (ACGS), ratified by the Dutch Society of Clinical Genetic Laboratory Specialists, and largely in agreement with the American College of Medical Genetics and Genomics (ACMG) guidelines [34–36]. Results underwent multidisciplinary review by 2 registered clinical laboratory geneticists, a hematologist, a coagulation laboratory specialist, and the clinical researcher of the study. We reported relevant variants of unknown significance (VUS; class 3), likely pathogenic (class 4), and clearly pathogenic (class 5) variants. Variants in both autosomal dominant and recessive disease genes were included, following the main inheritance described in the Online Mendelian Inheritance of Men (OMIM) database (Supplementary Table S3) [37].

2.4 | Clinical interpretation of targeted exome analysis

An 8-category scale was developed by discursive reasoning, integrating OMIM inheritance and ACGS classification, for an optimal interpretation of rare genetic variants (Table 1) [35,37]. Multiple variants in a single gene were hypothesized as *trans* and interpreted as compound heterozygous if patients had factor activity levels ≤ 5%, fibrinogen levels ≤ 250 mg/L, or had a proven compound heterozygous genotype prior to this study. Multiple variants in the same gene that could not be interpreted as compound heterozygous were categorized as "phase unknown," as no segregation analysis was performed. These variants were assigned a higher category compared with a single variant.

2.5 | Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics, version 29 (IBM, SPSS Inc). Descriptive statistics were used for patient characteristics, and values are reported as median with IQR if not mentioned otherwise. Statistical tests, including chi-squared test, Fisher's exact test, Mann–Whitney U-test, and Kruskall–Wallis test, were employed based on data characteristics. Correlation between coagulation factor activity levels and genetic variant category was tested with the Spearman rank correlation coefficient. Strength of the correlation coefficient (r) was categorized as nonexistent (<0.19), weak (0.20-0.39), moderate (0.40-0.59), strong (0.60-0.79), or very strong (>.80) [38]. Patients using medications potentially affecting coagulation factor activity levels, such as vitamin K antagonists and direct oral anticoagulants (DOACs), were excluded from statistical analyses pertaining to relevant coagulation factors. A result was considered statistically significant if $P < .05$.

TABLE 1 Categories of the 8-tier scale (0-7). This scale combines established mode of inheritance (Online Mendelian Inheritance of Men) with the 5-tier classification according to the practice guidelines of the Association for Clinical Genetic Science, with a description of the contents of each category. Case identification numbers (corresponding with Supplementary Table S3) are provided as an example and for clarification.

Severity of variant
Category 0
No class 3, 4, or 5 variants
Category 1
Class 3 heterozygous, AR
Category 2
Class 3 2 variants phase unknown, AR (cases 87 and 105)
Category 3
Class 3 homozygous
Class 3 compound heterozygous
Class 3 heterozygous, AD
Class 3 hemizygous (case 156) or hemizygous, XL (case 151)
Category 4
Class 3 2 heterozygous variants, AD (case 69)
Class 3 2 homozygous variants (case 105)
Class 4 heterozygous, AR
Class 4 and class 3 variants phase unknown, AR (case 24)
Category 5
Class 4 2 heterozygous variants phase unknown, AR (case 9)
Class 5 and class 3 variants phase unknown, AR (case 115)
Class 5 heterozygous, AR
Category 6
Class 4 homozygous
Class 4 compound heterozygous
Class 4 heterozygous, AD
Class 4 hemizygous, XL
Class 5 and class 3 compound heterozygous (cases 118 and 119)
Class 5 and class 4 variants phase unknown, AR (cases 8 and 90)
Class 5 2 heterozygous variants phase unknown, AR (case 77)
Category 7
Class 5 homozygous
Class 5 compound heterozygous (case 123)
Class 5 heterozygous, AD
Class 5 hemizygous, XL
Class 5 heterozygous and class 4 compound heterozygous (cases 120 and 121)

AR, autosomal recessive; AD, autosomal dominant; XL, X-linked.

3 | RESULTS

3.1 | General results of targeted exome analysis

Out of 263 RBiN participants, 167 gave permission for targeted exome analysis [2]. Mainly due to insufficient quality, genetic data of 156 patients were available (Figure 1). A total of 214 variants (1.4/patient) were identified in thrombosis and hemostasis genes, with 114 (53%) being unique. Of these 114 unique variants, 34 (30%) were novel. Due to the targeted exome analysis, it is uncertain whether large deletions (case identification numbers: 4, 9, 86, and 156) were novel or reported previously. Single nucleotide variants were responsible for 90% of the variants, and the remainder consisted of small (<20 bp) deletions or insertions (6%), gross deletions (3%), and small indels (1%). Most variants (n = 123; 57%) were classified as clearly pathogenic. Additionally, 40 (19%) likely pathogenic variants and 51 (24%) VUS were identified.

In 132 patients (85%), gene panel analysis yielded rare genetic variants corresponding to the patients' specific RBDs. Additional rare genetic variants were detected in genes from the gene panel in 36/132 (27%) patients with a genetic variant in the corresponding RBD gene. These variants were present in platelet disorder genes (n = 10), the *VWF* gene (n = 12), *F8* (n = 2), *PROS1* (n = 2), and other RBD genes (n = 12). Of these additional rare variants, 14 were classified as clearly pathogenic (37%), 8 likely pathogenic (21%), and 18 VUS (47%).

In 24 patients (15%), mainly with PAI-1 deficiency (n = 10) and hyperfibrinolysis (n = 7), no genetic variant was identified specific to their bleeding disorder. Other patients without a genetic variant matching their RBD had fibrinogen disorders (n = 2), FV (n = 2), FVII (n = 2), or FXI deficiency (n = 1). However, 13 variants in other genes were found in 9/24 patients (38%) that may contribute to a bleeding phenotype. These variants occurred in platelet disorder genes (n = 6), *VWF* (n = 3), or other RBD genes (n = 4).

The results of targeted exome analysis of all 156 patients are summarized in a heatmap (Figure 2), which combines assigned pathogenicity, inheritance, laboratory, and clinical data. Supplementary Table S3 provides an overview of all reported genetic variants.

3.2 | Laboratory phenotype

Coagulation factor activity levels (fibrinogen, FII, FV, FVII, FVIII, FX, FXI, FXIII, and A2AP) were measured and compared between RBDs. The resulting boxplots (Figure 3A–I) show markedly lower factor activity levels corresponding with the patients' individual RBD. Highlighted in Figure 3 are notable outliers with coagulation factor activity levels below the 95th percentile. Patients who used vitamin K antagonists, highlighted in green, had lower vitamin K-dependent coagulation factor activity levels. Furthermore, low FVIII activity levels were measured in 1 patient with a DOAC, most likely due to

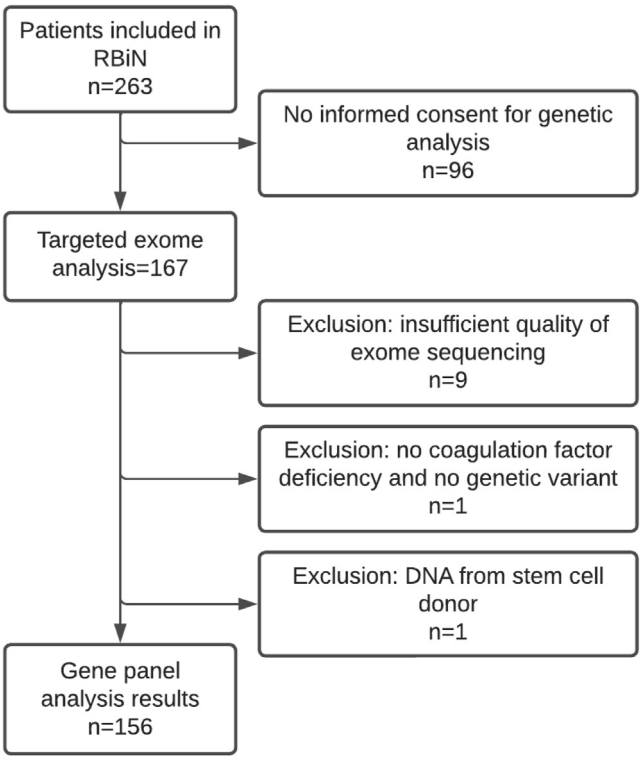


FIGURE 1 Flowchart of patient inclusion. RBiN, Rare Bleeding Disorders in the Netherlands.

assay interference. In addition, patients with FV Amsterdam had notably higher FV activity levels and markedly lower FVII activity levels, which may be explained by direct inhibition of FVII by high tissue factor pathway inhibitor levels [39].

For most coagulation factor deficiencies, a gradual decrease of category level was observed as coagulation factor activity levels normalized per RBD (indicated by color intensity, Figure 2). Overall, there was a strong to moderate correlation between factor activity levels and the designated genetic variant category of the corresponding RBD (Supplementary Figure S1A–H). Patients with FV Amsterdam were excluded from the analysis between *F5* and FV activity levels, and *F7* and FVII activity levels. When multiple fibrinogen genes were affected (n = 1), the variant of the highest category was used.

Strong correlations were observed between factor activity levels and category of genetic variant for fibrinogen genes (*FGA*, *FGB*, and *FGG;* n = 154; r = −0.65 [95% CI, −0.73 to −0.54]; P < .001) and *F7* (n = 146; r = −0.65 [95% CI, −0.74 to −0.54]; P < .001). In addition, we discovered a moderate correlation for *F2* (n = 148; r = −0.43 [95% CI, −0.56 to −0.29]; P < .001), *F5* (n = 150; r = −0.48 [95% CI, −0.56 to −0.34]; P < .001), *F11* (n = 154; r = −0.55 [95% CI, −0.66 to −0.43]; P < .001), *F13A1* (n = 131; r = −0.46 [95% CI, −0.59 to −0.31]; P < .001), and *SERPINF2* (A2AP; n = 154; r = −0.51 [95% CI, −0.62 to −0.38]; P < .001). Lastly, a weak correlation was found for *F10* (n = 148; r = −0.31 [95% CI, −0.45 to −0.15]; P < .001), which must be interpreted with caution, as only 5 patients (including 1 patient with FVII deficiency) with a *F10* variant were included in this study. Performing this analysis with the exclusion of VUS had no impact on the strength of the Spearman rank correlation coefficients.

3.3 | Clinical phenotype

Bleeding tendency varied across RBD groups, with overall high ISTH-BAT scores (range, 6-18; Table 2). ISTH-BAT scores were missing in 19 patients (12%). We report generally mild factor deficiencies in our population, except for FXIII deficiency. Diagnostic rationale was available for 110 patients (71%): patients were diagnosed because of bleeding symptoms in 38% (n = 42), aberrant coagulation profile on routine laboratory examination in 16% (n = 17), and in 46% (n = 51) because of an affected family member. As expected, median ISTH-BAT scores significantly differed between these groups, with the highest median (13 [IQR, 11-18]) in patients with bleeding symptoms compared with patients diagnosed because of an aberrant coagulation profile (median, 9 [IQR, 4.5-13.5]), or family members (median, 6.5 [IQR, 4-11]). Overall, ISTH-BAT scores were not significantly different between men and women, except in patients with FVII deficiency, where men had significantly lower (7.5) median ISTH-BAT scores than women (13). ISTH-BAT scores available in 3 children were 0, 3, and 24, respectively.

In total, 17/153 (11%) patients with RBDs were using antithrombotic medication at the time of inclusion, and data regarding antithrombotic use was missing in 3 patients. Six patients used vitamin K antagonists, 1 patient used a DOAC, 7 patients used aspirin, 2 patients used clopidogrel, and 1 patient used a combination of aspirin and clopidogrel. Median ISTH-BAT scores did not differ significantly between patients who used antithrombotics (median, 10 [IQR, 4-14]) and those who did not (median, 10 [IQR, 6-16]; P = .357). Interestingly, median ISTH-BAT scores for 7 patients who used a DOAC or vitamin K antagonists were significantly lower (median, 4 [IQR, 3-7]) compared with the remaining population (median, 11 [IQR, 6-15]; P = .014).

3.4 | Co-occurrence of multiple genetic variants

Supplementary Table S4 provides information for patients with co-occurrence of multiple genetic variants. Co-occurrence of multiple genetic variants did not significantly affect median ISTH-BAT scores compared with patients with a single genetic variant (both with median, 10 [IQR, 5-15]) and was not associated with bleeding grade (median grade, 2 [IQR, 0-3]). FV Leiden was present in 4 patients (2.6%), comparable with the prevalence in the overall Dutch population (2.9%) [40]. Our genetic data revealed no participants with a prothrombin G20210A polymorphism (prevalence in the Dutch population: 1.7%) [41]. Two patients with A2AP deficiency and a VUS in *PROS1* were noted. Overall, the median ISTH-BAT score for these 6 patients harboring a prothrombotic variant was 2.5 (range, 0-16), significantly (P = .036) lower than in patients without a prothrombotic variant (median ISTH-BAT, 10; range, 0-30).

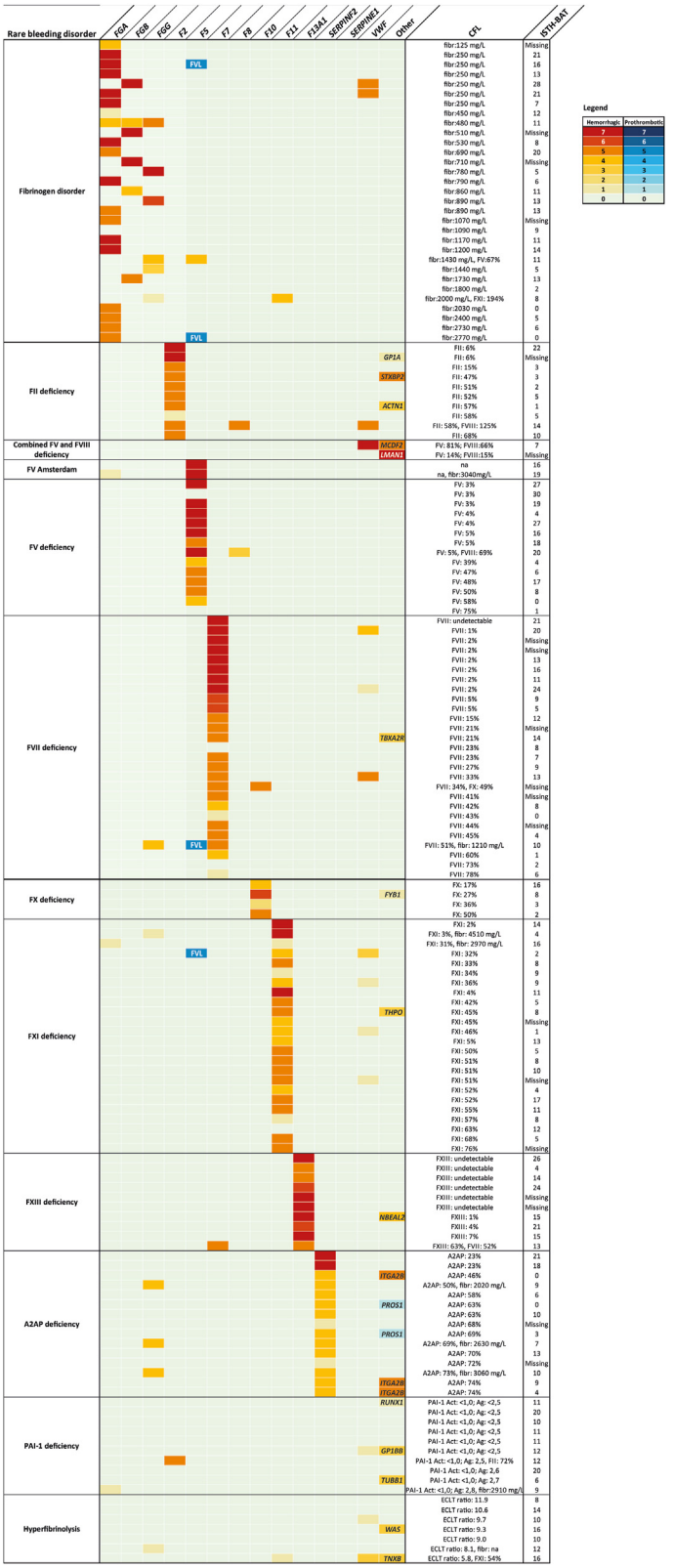


FIGURE 2 Heatmap that summarizes gene panel analysis by exome sequencing results of 156 patients. The genetic variants are color-coded based on their designated category as described in the Methods and Table 1. The legend of the color code is displayed on the right and shows colors for hemorrhagic genetic variants on the left and colors for prothrombotic genetic variants on the right. Each row in the heatmap represents 1 individual patient. Some genes were infrequently affected, and these genes are combined in the second-last column, "other genes," where the corresponding gene symbol is provided in the appropriate box. The factor (F)V Leiden variant is abbreviated as FVL. Cases are sorted by coagulation factor activity levels (CFLs) of the corresponding coagulation deficiency, starting from the top with the lowest CFL, and are displayed on the right side of the heatmap. Cases of plasminogen activator inhibitor type 1 (PAI-1) deficiency and hyperfibrinolysis are sorted by PAI-1 antigen and euglobulin clot lysis time (ECLT) ratio, respectively. Further information on all reported genetic variants is provided in Supplementary Table S3. A2AP, alpha-2-antiplasmin; Fibr, fibrinogen; FII/FV/FVII/FX/FXI/FXIII, factor II/V/VII/X/XI/XIII; ISTH-BAT, International Society on Thrombosis and Haemostasis Bleeding Assessment Tool; NA, not available.

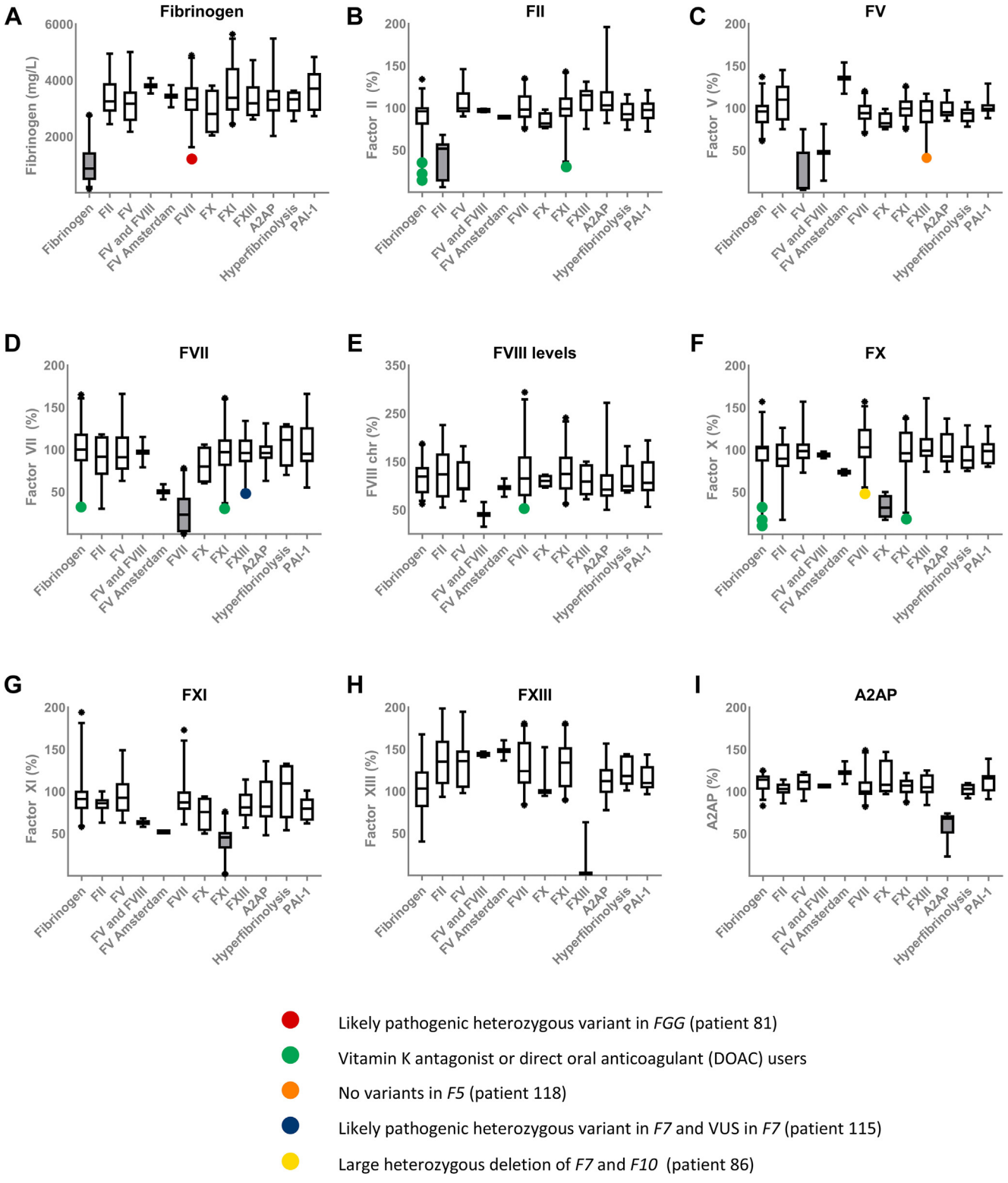


FIGURE 3 (A–I) Boxplots of factor activity levels measured in different rare bleeding disorder groups. Individual outliers with corresponding patient numbers (Supplementary Tables S3 and S4) are marked with different colors. A2AP, alpha-2-antiplasmin; FII/FV/FVII/FX/FXI/FXIII, factor II/V/VII/X/XI/XIII; PAI-1, plasminogen activator inhibitor type 1; VUS, variants of unknown significance.

TABLE 2 Overview of demographic data of included Rare Bleeding Disorders in the Netherlands patients.

	Patients	Female		Age, y	Coagulation factor activity levels	ISTH-BAT
Rare bleeding disorder	*n*	*n*	(%)	Median (IQR)	Median (IQR)	Median (IQR)
Fibrinogen deficiency	31	21	68	44 (32-56)	860 mg/L (450-1440)	11 (6-13)
FII deficiency	10	6	60	54 (37-68)	52% (13%-58%)	7 (3-11)
FV Amsterdam	2	1	50	40 (31-48)	Not available	18 (17-18)
FV deficiency	14	9	64	41 (31-62)	5% (4%-49%)	17 (4-22)
Combined FV and FVIII	2	1	50	21 (16-26)	FV: 48%; FVIII: 41%	7
FVII deficiency	27	13	48	44 (30-56)	23% (2%-43%)	9 (6-14)
FX deficiency	4	3	75	51 (33-58)	32% (20%-47%)	6 (2-14)
FXI deficiency	24	15	63	56 (36-67)	46% (32%-52%)	8 (5-12)
FXIII deficiency	10	3	30	47 (19-54)	Undetectable (undetectable to 5%)	15 (13-23)
A2AP deficiency	15	11	73	50 (36-66)	68% (23%-74%)	9 (4-12)
PAI-1 deficiency	10	10	100	43 (26-51)	Act: <1.0 ng/mL (<1.0 to <1.0) Ag: 2.3 ng/mL (1.0-2.6)	11 (10-14)
Hyperfibrinolysis	7	6	86	62 (47-74)	ECLT ratio: 9.3 (8.1-10.6)	12 (10-16)
Total	156	99	63	46 (32-62)	Not applicable	10 (5-15)

Mean and range are used instead of median and IQR in patients with FV Amsterdam and combined FV and FVIII deficiency.

A2AP, alpha-2-antiplasmin; Act, activity; Ag, antigen; ECLT, euglobulin clot lysis time; FII/FV/FVII/FX/FXI/FXIII, factor II/V/VII/X/XI/XIII; ISTH-BAT, International Society on Thrombosis and Haemostasis Bleeding Assessment Tool; PAI-1, plasminogen activator inhibitor type 1.

3.5 | Variants in the von Willebrand gene

In total, 14 patients carried 15 variants in the *VWF* gene, with 10 being VUS. Although we observed a higher ISTH-BAT (median, 13 [IQR, 8-21]) compared with patients without *VWF* variants (median, 10 [IQR, 5-14]), this difference was not statistically significant (P = .137). Additionally, no significant differences were observed between patients with and without *VWF* variants for FVIII activity levels, VWF:Ag, VWF:RCo, platelet count, PFA-epinephrine (EPI), or PFA-adenosine diphosphate (ADP) (Supplementary Table S5A). After selecting patients with likely and clearly pathogenic variants, ISTH-BAT scores remained nonsignificantly higher in patients with a variant in *VWF* (median, 14 [IQR, 10-25]; P = .07), with no significant effect on laboratory parameters (Supplementary Table S5B). No correlation between *VWF* and VWF:Ag or VWF:RCo could be demonstrated in this cohort (data not shown).

3.6 | Platelet disorder variants

In 15 patients (10%), 16 variants in platelet disorder genes were found. Most of these variants were VUS (n = 11), with the exception of a likely pathogenic variant in *NBEAL2* and clearly pathogenic variants in *STXBP2* (n = 1) and *ITGA2B* (n = 3). None of these 15 patients displayed thrombocytopenia (defined as platelet count < 150 × 10^9/L). Platelet count did not significantly differ between patients with a platelet disorder variant (median, 237 × 10^9/L [IQR, 219-274]) and those without (243 × 10^9/L [IQR, 212-283]; P = .787).

PFA-closure time (CT) results were frequently missing (31%-43%). In 15 patients with platelet disorder variants, missing data approached 50% (with 3/5 PFA-CT missing in patients with likely or clearly pathogenic variants). PFA-EPI (median, 129 [IQR, 100-175]) and PFA-ADP (median, 95 [IQR, 75-135]) in patients with platelet disorder variants did not differ significantly from patients without such a variant (PFA-EPI median, 134 [IQR, 116-167]; P = .576; PFA-ADP median, 104 [IQR, 91-124]; P = .54). Patients who used aspirin, clopidogrel, or NSAIDs (n = 16) were excluded from this analysis. No platelet disorder variants were found in patients with thrombocytopenia (n = 4; range, 83-138 × 10^9/L) or in patients previously diagnosed with thrombopathy (n = 2).

4 | DISCUSSION

This study presents results from targeted exome analysis in a Dutch RBD population, integrating genetic data with bleeding phenotype and laboratory data. Consistent with previous studies that report a high diagnostic sensitivity of HTS in well-defined bleeding disorders, targeted exome analysis revealed rare class 3, 4, or 5 variants in the corresponding RBD gene in most patients (85%) [18–21,42,43]. An 8-tier genetic variant category system incorporating established inheritance (OMIM) with pathogenicity according to ACGS guidelines showed significant correlations with factor activity levels [34–36].

Co-occurrence of genetic variants in thrombosis and hemostasis-related genes was identified in nearly a quarter of participants, a frequency higher than observed in previous gene panel studies

[18–20]. Rare variants in diagnostic-grade genes for rare bleeding, thrombotic, and platelet disorders have previously been discovered in 9% of the population, constituting approximately a third of these co-occurrences [44]. Furthermore, this study reports VUS in RBDs and other thrombosis and hemostasis genes with an autosomal dominant inheritance pattern, potentially explaining the observed high frequency. These findings may suggest that an additional genetic defect is required for a bleeding disorder to manifest, specifically in heterozygous carriers. However, co-occurrence of multiple genetic variants had no significant effect on median ISTH-BAT scores. Notably, overall high ISTH-BAT scores (median, 10 [IQR, 5-15]) were observed in the RBiN population.

Additional genetic variants were predominantly identified in *VWF* and platelet disorder genes, with two-thirds of cases involving VUS. Previous research in patients with von Willebrand disease reported additional (mild) coagulation defects in a considerable portion of patients (10%-36%) [45–47]. Although ISTH-BAT was higher in these patients, this increase did not reach statistical significance. It is noteworthy that VWF:Ag and VWF:RCo in our cohort did not fall below 50%. However, due to variations in timing of blood sample collection, diurnal fluctuations in VWF:Ag levels, with midday peak levels, may play a role [48]. Additionally, prior studies in UK Biobank participants with pathogenic variants in *VWF* showed no clear effect on odds ratio for bleeding [44]. Therefore, it may reasonably be assumed that presence of a nonpathogenic *VWF* variant without low VWF levels has no clear impact on bleeding phenotype in the RBiN population. Moreover, platelet disorder variants were not associated with prolonged PFA-CT or decrease in platelet count. Because of the small sample size, the prevalence of VUS, and frequently missing PFA-CT results, drawing definitive conclusions is impossible. Specific evaluation of platelet function in patients with platelet disorder variants may offer some insights into the clinical relevance of such variants.

Median ISTH-BAT scores of 6 patients with a prothrombotic variant were lower than the overall RBiN population (2.5 vs 10). Co-occurrence of a prothrombotic variant may ameliorate bleeding risk in patients with RBDs, as these variants have been associated with lower annual bleeding rates and delayed onset of first bleeding in patients with hemophilia [49,50]. Investigating prothrombotic variants (including polymorphisms) in a larger cohort of RBDs would be valuable in understanding their potential impact on RBDs.

Patients with anticoagulants had lower median ISTH-BAT scores compared with the rest of the RBiN population. Patients taking anticoagulants typically have an underlying medical condition necessitating their use, often due to a history of arterial or venous thrombosis. Consequently, their phenotype may lean more toward the thrombotic end, which may translate into lower bleeding scores. We observed that 3 out of 7 patients (43%) using DOACs or coumarin derivatives had a history of thrombosis compared with 12 out of 133 patients (36%) in the remaining RBiN population. Although these numbers are small, the difference was statistically significant (P = .02). Four patients had missing data in this analysis. Unfortunately, the duration of anticoagulant usage was not available.

The bleeding phenotype in RBD individuals appears to result from a complex interplay of laboratory parameters, genetic, and external factors [2,17,19,20,44]. We believe that gene panel analysis through exome sequencing offers an advantage over single-gene analysis. Targeted exome analysis may improve tailored patient treatment, emphasized by the number of combined deficiencies found in this study. However, the clinical relevance of co-occurrence of multiple variants in thrombosis and hemostasis genes needs careful consideration within the context of the patient's phenotype and prevalence within the normal population [24]. Multidisciplinary collaboration between clinical geneticists, laboratory specialists, and clinicians is therefore required. In the future, analyzing hemostasis in patients with multiple co-occurring variants, including prothrombotic ones, could potentially be enhanced by incorporating global hemostasis assays alongside assessments of primary hemostasis.

No explanatory genetic variants were identified for patients with PAI-1 deficiency and hyperfibrinolysis. In half of these patients, VUS in other genes was identified, with the addition of 1 clearly pathogenic variant in *F2*. Remarkably, no genetic variants were found in *SERPINE1*, previously linked to PAI-1 deficiency [51]. Fibrinolytic abnormalities may account for a substantial portion of patients with bleeding of unknown origin [27,52], and the diagnostic yield of HTS in patients with unexplained bleeding is notoriously low (3%-17%) [19–22,42]. Fibrinolytic disorders are difficult to diagnose because clinically validated assays to detect a hyperfibrinolytic state are lacking, and therefore, assessment of fibrinolysis is not (yet) a standard element in the work-up of bleeding disorders [27,52,53]. Unfortunately, WES is limited to exome sequencing only, excluding information on intronic 4G/5G polymorphisms that can influence and downregulate circulating PAI-1 activity levels, possibly explaining the lack of genetic confirmation [54]. Whole genome sequencing in a larger cohort of patients with PAI-1 deficiency and hyperfibrinolysis may help identify new causative genetic variants and could potentially improve the diagnosis of fibrinolytic disorders.

Our study has several limitations. First, the cross-sectional design and relatively small sample size are inherent to researching rare disorders. Secondly, gene panel analysis by exome sequencing fails to capture deep intronic variants and is not ideal for calling (large) indels. However, a standardized gene panel HTS reduces risks of incidental findings and has become a standard diagnostic approach in the field of RBDs and beyond [20,25,55]. Furthermore, ACMG-specific variant codes are not available, as ACGS guidelines were followed in the reporting of variants according to national practice. Moreover, the collected data were insufficient to certainly determine the number of families included in this study, as no segregation analysis was performed, and it was not recorded which patients belonged to the same family prior to pseudonymization. Overall, RBiN patients presented with high bleeding scores despite relatively mild factor deficiencies, suggesting a potential selection bias of severe patients with RBDs in Dutch HTCs. Nevertheless, the RBiN study population likely reflects the actual RBD population treated in HTCs. Additionally, ISTH-BAT scores were based on current status, which may have resulted in higher overall bleeding scores. Unfortunately, presence of

hypermobility symptoms was not routinely assessed in our study. Lastly, exclusion of variants with population frequencies >1% means that common variants with known functional effects were not considered [56]. Future analyses are planned to explore the effect of common variants within the RBiN population.

5 | CONCLUSIONS

In summary, targeted exome analysis reveals diverse genetic profiles in Dutch patients with RBDs, and most patients harbored rare genetic variants specific to their RBD. Co-occurrence of variants in multiple genes existed in a quarter of patients but did not affect median ISTH-BAT scores. Furthermore, an overall significant correlation exists between genetic variant category of an RBD gene (based on inheritance and pathogenicity) and corresponding factor activity levels. WES with a multigene panel may offer an advantage over single-gene analysis, especially considering personalized treatment in complex patients with RBDs. Further studies employing cosegregation analysis and functional studies within families are necessary to determine the impact of co-occurrence of multiple variants on bleeding severity. Global hemostatic assays, covering a more overall view of parts of the hemostatic system, may provide additional valuable insights in this regard.

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AUTHOR CONTRIBUTIONS

M.H.C., P.L.d.E., I.C.K., K.M., L.N., N.v.E., R.E.G.S., W.L.v.H., and S.E.M.S. are members of the steering committee that designed the Rare Bleeding Disorders in the Netherlands (RBiN) study and are delegates of all Dutch Hemophilia Treatment Centers and the patient society; N.M.A.B. is head of the department of Hematology in the Radboud University Medical Center and head of the RBiN project management team; J.L.S. interviewed the patients; S.S. analyzed the blood samples; A.S. and J.W. analyzed the genetic data; S.P.E.W. analyzed the data; S.P.E.W., S.E.M.S., and W.L.v.H. prepared the manuscript; S.R. cowrote methods on laboratory assessment; all authors revised the manuscript and gave final approval.

RELATIONSHIP DISCLOSURE

K.M. reports speaker fees from Bayer and Alexion, participation in a trial steering committee for Bayer, consulting fees from UniQure, participation in data monitoring, and endpoint adjudication committee for Octapharma. M.H.C. is coordinator of Erasmus Medical Center as a Health Care Provider within the European Reference Network (ERN) for rare hematological diseases EuroBloodNet and (co)leader of the local Erasmus Medical Center Expert Centers for Rare Bleeding Disorders and Sickle Cell and Thalassemia Comprehensive Care Center. She has received investigator-initiated research and travel grants as well as speaker fees over the years from the Netherlands Organization for Scientific Research (NWO [Nederlandse Organisatie voor Wetenschappelijk Onderzoek]), the Netherlands Organization for Health Research and Development (ZonMw [ZorgOnderzoek Nederland Medische Wetenschappen]), the Dutch "Innovatiefonds Zorgverzekeraars," Baxter, Baxalta, Shire, Takeda, Pfizer, Bayer Schering Pharma, CSL Behring, Sobi Biogen, Novo Nordisk, Novartis, and Nordic Pharma, and has served as a steering board member for Roche, Bayer, and Novartis. All grants, awards, and fees go to the Erasmus Medical Center as an institution. R.E.G.S. reports grants from Bayer, Baxalta, Pfizer, and Novo Nordisk outside the submitted work. W.L.v.H. reports financial support from Takeda, Bayer, Sobi, and CSL Behring, and funding from Takeda and Bayer for Enzyre BV. The remaining authors declare no competing financial interests.

DATA AVAILABILITY

All rare genetic variants discovered with the 156-gene panel analysis by exome sequencing may be found in Supplementary Table S3. All variants will be uploaded to the ClinVar database. Additional data are available upon reasonable request from the corresponding author, Saskia Schols (saskia.schols@radboudumc.nl).

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SUPPLEMENTARY MATERIAL

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