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**Rare Bleeding Disorders: Insights into Clinical Presentation, Laboratory
Phenotype, and Genetic Characterization**

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PhD candidate: Samin MOHSENIAN

Matr. N. R13785

ORCID no. <https://orcid.org/0000-0001-5004-4456>

Supervisor: Prof. Flora PEYVANDI

PhD Program Director: Prof. Mario Salvatore Clerici

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Summary

Rare bleeding disorders (RBDs) account for approximately 3–5% of all inherited bleeding conditions. RBDs include deficiencies of fibrinogen, factor (F) II, V, VII, X, XI, and XIII, most of which follow an autosomal recessive inheritance pattern. These disorders typically arise from pathogenic variants in the genes encoding specific coagulation factors. Among RBDs, congenital fibrinogen disorders (CFDs) account for ~8% of RBDs and are classified based on fibrinogen activity and antigen levels into quantitative (afibrinogenemia, hypofibrinogenemia) and qualitative (dysfibrinogenemia, hypodysfibrinogenemia) deficiencies. This thesis represents an innovative and comprehensive effort to advance the understanding of RBDs, integrating genetic analyses, standardized clinical assessments, and laboratory phenotyping, and contributing to a major step forward in the field.

(I) In the first chapter, we analyzed the genotype and laboratory phenotype of 526 RBD cases, one of the largest cohorts to date. In our study, FVII deficiency was the most common (23%), while FII (prothrombin) and FV+FVIII deficiencies were the rarest (6%). Among 257 unique variants identified, 86% were predicted pathogenic and 11% were novel. Missense variants predominated (57%), mainly affecting exons encoding catalytic domains (48%). (II) In the second chapter, we compared the adequacy of two bleeding score systems (ISTH-BAT and EN-RBD-BSS) in identifying patients with CFDs, and analyzed their correlation with fibrinogen levels and clinical severity grades. Our findings suggest that, alongside ISTH-BAT, the EN-RBD-BSS can also be useful for identifying CFD patients. Both tools demonstrated good sensitivity for detecting fibrinogen deficiency, and bleeding severity scores correctly classified clinical severity in nearly two-thirds of cases. (III) In the third chapter, a total of 166 CFD cases were comprehensively characterized using the Prospective Rare Bleeding Disorders Database. This large multicenter study offered valuable insights into the clinical, laboratory, and genetic profiles of CFDs patients. Bleeding severity grades aligned with the established factor activity thresholds in nearly half of the cases with quantitative fibrinogen defects. (IV) In chapter four, we addressed gynecologic and obstetric complications in women with congenital fibrinogen disorders. Our cohort included 59 women with CFDs, encompassing 70 pregnancies in 32 individuals. Heavy menstrual bleeding was most common in afibrinogenemia, affecting 75% of cases. Postpartum hemorrhage (36%) and miscarriage (23%) rates were notably high. No significant difference in obstetric risk was found between dysfibrinogenemic women with and without hotspot variants. These results highlight the need for early diagnosis and possible prophylaxis, even in asymptomatic cases.

Chapter 0

An introduction to rare bleeding disorders

Hemostasis

Hemostasis is a regulated process that maintains blood fluidity under physiological conditions and ensures rapid cessation of bleeding following vascular injury. It involves three phases: primary hemostasis, characterized by platelet adhesion and aggregation; secondary hemostasis, during which coagulation factors generate thrombin leading to fibrin formation; and fibrinolysis, the final phase responsible for clot resolution [1]. The coagulation cascade, a central component of secondary hemostasis, relies on the sequential activation of plasma coagulation factors. Disruptions in this cascade, due to genetic or acquired defects, result in bleeding disorders of variable severity [2].

Rare bleeding disorders

Rare bleeding disorders (RBDs) account for approximately 3–5% of all inherited bleeding conditions [3]. RBDs include deficiencies of fibrinogen (factor I), prothrombin (II), and factors (F)V, VII, X, XI, XIII and vitamin K–dependent coagulation factor deficiency (VKCFD), most of which follow an autosomal recessive inheritance pattern [4]. These disorders are especially prevalent in populations with high rates of consanguinity [5]. Their clinical manifestations range from asymptomatic abnormalities to severe spontaneous bleeding, depending on the factor involved and its residual activity. RBDs typically arise from pathogenic variants in the genes encoding specific coagulation factors, most of which affect catalytic or functional domains, leading to reduced synthesis, impaired secretion, or loss of activity of the respective proteins [4]. The combined deficiency of FV and FVIII is caused by defects in the *LMAN1* and *MCFD2* genes, which encode proteins responsible for transportation of both factors from endoplasmic reticulum to the Golgi apparatus [6]. VKCFD is the result of genetic mutations in the genes encoding enzymes critical for post-translational modifications and vitamin K metabolism, specifically γ -glutamyl carboxylase and vitamin K epoxide reductase [7].

Congenital fibrinogen deficiencies

Among RBDs, congenital fibrinogen disorders (CFDs) account for ~8% of RBDs and are classified based on fibrinogen activity (Fg:C) and antigen levels (Fg:Ag) into quantitative (afibrinogenemia, hypofibrinogenemia) and qualitative (dysfibrinogenemia, hypodysfibrinogenemia) deficiencies [8, 9]. Clinical manifestations range from asymptomatic to severe bleeding, with occasional thrombotic events [10, 11]. Afibrinogenemia causes undetectable fibrinogen levels, leading to a severe bleeding

and, less commonly, thrombosis [12-14]. Hypofibrinogenemia, with proportionally reduced Fg:C and Fg:Ag, is usually associated with mild bleeding and rare thrombotic complications [15, 16]. In dysfibrinogenemia, Fg:C is low while Fg:Ag remains normal; most patients are asymptomatic, but some present with bleeding or thrombosis [10, 17, 18]. Hypodysfibrinogenemia involves disproportionately reduced Fg:C and Fg:Ag, and may present with more severe clinical features, including thrombosis [15]. Genetic variants of *FGA*, *FGB* and *FGG* are useful for the correct diagnosis and to identify its types and subtypes of CFDs [19].

Bleeding assessment tools

Assessment of clinical history is essential for guiding the diagnostic workup of patients with suspected bleeding disorders. To support this process and standardize symptom evaluation, bleeding assessment tools (BATs) have been developed since 1992 [20]. In 2010, the International Society on Thrombosis and Haemostasis (ISTH) proposed the ISTH-BAT, which was first validated for clinical screening of suspected von Willebrand disease (VWD) and for predicting bleeding risk [21, 22]. The ISTH-BAT bleeding score is considered normal if 0–3 in adult males, 0–5 in adult females, and 0–2 in children. Thus, a positive or abnormal BS is defined as ≥ 4 in adult males, ≥ 6 in adult females, and ≥ 3 in children, thresholds that help distinguish individuals with bleeding disorders from healthy subjects [23]. According to a systematic review, the pooled sensitivity and specificity for diagnosing VWD were estimated at 75% (95% CI, 66–83) and 54% (95% CI, 29–77), respectively [24]. However, only a limited number of patients with RBDs were evaluated using these tools, and no conclusive results have been obtained [25, 26].

According to the study by the EN-RBD, RBD patients can be classified as asymptomatic, grade I, grade II, or grade III based on locations, potential clinical impact and spontaneity of bleeding [27]. More recently and within the same project, Palla et al developed a novel bleeding score system (BSS) dedicated to RBDs (EN-RBD-BSS, from now on), which enlists previous bleeding symptoms, frequency, spontaneity, extent, localization and relationship with prophylaxis and acute treatment [28]. The cut-off value for an abnormal EN-RBD-BSS is ≥ 1.5 for men, women and children. This new BSS has been shown to be useful in identifying patients who have to be followed for further laboratory investigations.

Although the utility of such BS tools has been examined in cohorts including all RBDs [26, 27], no studies have been conducted specifically on CFD because of its low prevalence, which prevents large cohorts from being available.

Gynecological and obstetrical complications in women affected with CFD

Women and girls with RBDs face increased risks of adverse outcomes due to physiological hemostatic challenges during their reproductive years. Gynecological and obstetrical complications were reported in ~20% of women with all RBDs [8].

In CFDs, heavy menstrual bleeding (HMB) is often the most frequent, and in some cases the only, clinical manifestation [29, 30]. Pregnancy presents further complications, as fibrinogen is essential for placental maintenance, early trophoblast proliferation, and implantation, all of which are critical for a successful pregnancy [31, 32].

Women with severe CFD face a high risk of miscarriage and bleeding during pregnancy [33]. In afibrinogenemia and severe hypofibrinogenemia, fibrinogen concentrate should be started as soon as pregnancy is confirmed and maintained at ≥ 100 mg/dL throughout gestation [34, 35]. Miscarriage rates in women with moderate hypofibrinogenemia or dysfibrinogenemia, especially those with prothrombotic variants, range from 20% to 50%, notably higher than the global average of ~15% [36-39].

Despite these potential complications, data on gynecological and obstetric outcomes in individuals with CFDs remain scarce. Understanding the impact of these disorders on pregnancy, miscarriage risk, and PPH is crucial for improving clinical management and developing preventive strategies.

Aims

This three-year PhD thesis aimed to provide new insights into the clinical presentation, laboratory phenotype, and genetic characterization of RBDs. It represents an innovative and comprehensive effort to advance the understanding of RBDs, combining large-scale genetic analyses, standardized clinical assessment tools, and prospective patient data. By integrating molecular, clinical, and demographic information across diverse populations, this work significantly enhances the understanding of the diagnosis, characterization, and management of RBDs, thereby contributing to a major advancement in the field.

Chapter I focused on the genotype and laboratory phenotype of a large, international cohort of patients with RBDs, primarily from Europe and Asia. The main objective was to explore the genetic spectrum of RBDs using one of the largest cohorts reported to date, identifying the most common variants and variant types, and assessing the predicted pathogenicity of all identified variants. This work aimed to enhance our understanding of the molecular basis of RBDs.

Chapter II evaluated the diagnostic utility of two bleeding assessment tools, ISTH-BAT and EN-RBD-BSS, in a large, well-characterized cohort of patients with CFDs. The chapter also examined the correlation between BAT scores, plasma fibrinogen levels, and clinical severity classification.

Chapter III presented a cross-sectional study conducted within the Prospective Rare Bleeding Disorders Database (PRO-RBDD), aiming to describe the clinical, laboratory, and genetic features of a defined group of patients with CFDs. The database includes detailed demographic and clinical information from birth to enrollment, offering insights into the natural history and medical management of these patients. Additionally, this chapter aimed to assess the association between factor activity levels and clinical severity, validating previous findings from the EN-RBD study in a broader and more diverse population. It also explored potential differences in the age at diagnosis among the various subtypes of fibrinogen deficiency.

Chapter IV aimed to investigate the rate and spectrum of gynecologic and obstetric complications in women and girls with CFDs, using data from the PRO-RBDD.

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Chapter I

The genetic spectrum of rare bleeding disorders

Samin Mohsenian¹, Omid Seidizadeh², Andrea Cairo², Roberta Palla¹, Marzia Menegatti², Flora Peyvandi^{1,2}

1. Università degli Studi di Milano, Department of Pathophysiology and Transplantation, Milan, Italy
2. Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center and Fondazione Luigi Villa, Milan, Italy

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Abstract

Background

Rare bleeding disorders (RBDs) account for almost 3-5% of bleeding disorders. Because of their rarity, phenotype and genotype analyses have been conducted only on a limited number of cases.

Aims

To characterize the laboratory phenotype and genetic spectrum of a large international cohort of RBDs.

Material and methods

A total of 807 individuals suspected to have RBDs were collected (2001-2020). The severity of RBDs was assessed based on plasma clotting factor activity levels. Gene sequencing was performed to confirm the diagnosis. Novel variants were assessed using in-silico prediction tool (CADD and REVEL scores).

Results

After excluding 46 subjects with normal phenotype and genotype, 761 cases with RBDs from 19 countries were included. Of these, 526 had coagulant activity levels below the normal range with identified variants. FVII deficiency was the most frequent (23%), while FII and compound FV+FVIII (6%) deficiencies were the rarest. The majority of cases (86%) had an autosomal recessive pattern and among their heterozygous cases (22%), 8% were severely affected, suggesting that the second expected variant was not identified. No causative variant was identified in 4% of cases. Among the identified 257 unique variants, 86% were predicted to be pathogenic and 11% were novel. Missense variants were identified in 57% of cases, excluding cases of afibrinogenemia and compound FV+FVIII deficiencies. The majority (48%) affected exons encoding catalytic domains.

Conclusion

In this large group of RBDs, missense variants were the most prevalent, primarily affecting the catalytic domains of proteins, except in cases of afibrinogenemia and FV+FVIII deficiencies.

Keywords

Blood coagulation factor deficiencies, rare diseases, genetic, genotype

Introduction

Congenital rare bleeding disorders (RBDs) are due to inherited defects in coagulation factors, including fibrinogen, factor (F)II, FV, combined FV and FVIII, FVII, FX, FXI, FXIII, and vitamin K-dependent coagulation factor deficiency (VKCFD) [1]. RBDs typically result from variants in the genes encoding the relevant coagulation factors, with most affecting the catalytic domains [2]. The combined deficiency of FV and FVIII is caused by defects in the *LMAN1* and *MCFD2* genes, which encode proteins responsible for transportation of both factors from endoplasmic reticulum to the Golgi apparatus [3]. VKCFD is the result of genetic mutations in the genes encoding enzymes critical for post-translational modifications and vitamin K metabolism, specifically γ -glutamyl carboxylase and vitamin K epoxide reductase [4].

RBDs account for approximately 3-5% of bleeding disorders with a prevalence of 1:500,000 in FVII deficiency to 1 in 2–3 million for FII and FXIII deficiencies [5, 6]. There is a significant correlation between the coagulation factors activity levels and the severity of clinical bleeding in cases of fibrinogen, FX, and FXIII deficiencies, as previously reported [7]. Conversely, a less evident correlation with clinical bleeding severity in FV and FVII deficiencies has been documented, and the activity level of FXI is not a reliable predictor for clinical bleeding severity [7, 8]. The genetic inheritance pattern of RBDs is predominantly autosomal recessive, except for dysfibrinogenemia, hypofibrinogenemia, and hypodysfibrinogenemia, which are autosomal dominant, as well as a few cases of FXI deficiency, which may exhibit a dominant transmission [5]. Accurate diagnosis and development of appropriate treatments for RBDs require a comprehensive understanding of phenotype and molecular characterization of the RBDs [9].

In low resources countries, where individuals with coagulation disorders seldom survive into adulthood due to insufficient management, the primary measures for preventing the birth of affected children involve identifying carriers and conducting prenatal diagnosis [9, 10]. However, due to the scarcity of RBDs, current knowledge of their genetic backgrounds is still limited. In recent years, an increasing number of data on patients with RBDs has been collected at both national and international levels through data collection programs and projects within the framework of the World Federation of Hemophilia (WFH) annual global survey, the European Network of Rare Bleeding Disorders (EN-RBD), and the Prospective Rare Bleeding Disorders Database (PRO-RBDD). Nevertheless, the genetic analyses have been conducted only on a small subset of patients [7, 9, 11–15]. Nearly 1,850 unique genetic variants associated with RBDs have been reported in the available databases [16–18].

In this study, we characterized the genotype and laboratory phenotype of a large cohort of 761 patients with the RBDs worldwide, mainly from Europe and Asia. We aimed primarily to investigate the genetic spectrum of RBDs using the largest cohorts reported to date, identifying the most common variants, and variants types, as well as predicting the pathogenicity of all identified variants. This study might shed light on our understanding of the molecular basis of RBDs.

Materials and methods

Data collection

A total of 807 individuals from 19 countries, including those suspected of having RBDs and their relatives, were registered at their respective countries' Hemophilia Treatment Centers. Their samples were sent to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico) for genetic analysis between 2001 and 2020. Written informed consent was obtained from each enrolled individual or their parents following approval by the ethical review board of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy.

Phenotypic assays

Hemostatic Factor activity levels were assayed for all included individuals using ACL TOP IL, ACL 10000 IL, ACL 7000 IL, BCT Siemens, BCT Dade Behring, SYSMEX CA1500 and Thrombolyzer and STAR. The laboratory phenotype diagnosis was based on low levels of coagulant activity. According to the Standardization Committee of the International Society on Thrombosis and Hemostasis (ISTH), the proposed severity of RBD based on their factor activity levels has been previously reported (Suppl, Table S1) [19].

Genetic assay

Genomic DNA was extracted using standard methods [20]. Using polymerase chain reaction, genomic DNA was amplified under standard conditions for each coagulation factor's gene, and the amplified fragments were directly sequenced using sanger sequencing. The primers were designed to amplify regions encompassing approximately ± 10 base pairs upstream and downstream of the target site to ensure accurate sequencing coverage. The Human Gene Mutation Database (<https://www.hgmd.cf.ac.uk/ac/index.php>), the Leiden Open Variation Database (<https://www.lovd.nl/3.0/home>) and the Human fibrinogen database presentation (<https://site.geht.org/base-fibrinogene/>) were used to find the already reported variants associated with coagulation deficiencies. Novel variants were assessed using in silico prediction tools, including Combined Annotation Dependent Depletion (CADD, <https://cadd.gs.washington.edu/>) and Rare

Exome Variant Ensemble Learner (REVEL) through the Ensembl Variant Effect Predictor (VEP). The qualitative prediction is determined based on these scores, where substitutions with a CADD score of ≥ 20 are labeled as deleterious and all other substitutions are labeled as tolerated. The REVEL score for a missense variant ranges from 0 to 1, with scores ≥ 0.5 considered more likely to be pathogenic. The minor allele frequency (MAF) of each identified variant was extracted from the Ensembl Variant Effect Predictor (VEP). As part of the study, we utilized the Genome Aggregation Database (gnomAD) v4.1 to determine the population frequency of identified variants and to find the rare pathogenic variants with the highest MAF in the general population (<https://gnomad.broadinstitute.org/>). Notably, variants with a MAF greater than 1% were excluded, as they are considered polymorphic. The gnomAD v.4.1 is a resource developed by an international coalition of investigators that compiled and harmonized exome and genome sequencing data from 807,162 individuals.

Statistical analysis

The analyses were conducted using RStudio 2022.7.1 and Excel 2016. Values are reported as median (range); for analysis purposes, all undetectable values were considered to be zero.

Results

Population

This study included 807 individuals investigated for RBDs, of whom 46 were excluded due to normal phenotype and genotype results (Figure 1A). Of the remaining 761 investigated cases, 526 had coagulant activity levels below the normal range and were genetically characterized (Figure 1A), whereas in 20 (4%) cases, no genetic variants were identified. Therefore, the total analyses were performed on the 526 cases (378 probands and 148 relatives) with coagulant activity below the normal range. Our population included 263 females and 263 males, evenly distributed, representing individuals from 19 different countries. The majority of patients ($n = 390$) were from Italy and Iran, which may reflect the variable prevalence of consanguineous marriages across regions and the predominantly recessive inheritance pattern of most rare bleeding disorders (Suppl, Figure S1). In our cohort, FVII deficiency was the most frequent disorder, affecting 23% of studied individuals, followed by fibrinogen (20%) and FX (16%) deficiencies. In contrast, the rarest disorders were FII and compound FV+FVIII deficiency, each being found only in 6% of the population, followed by FXI with a frequency of 7% (Figure 2).

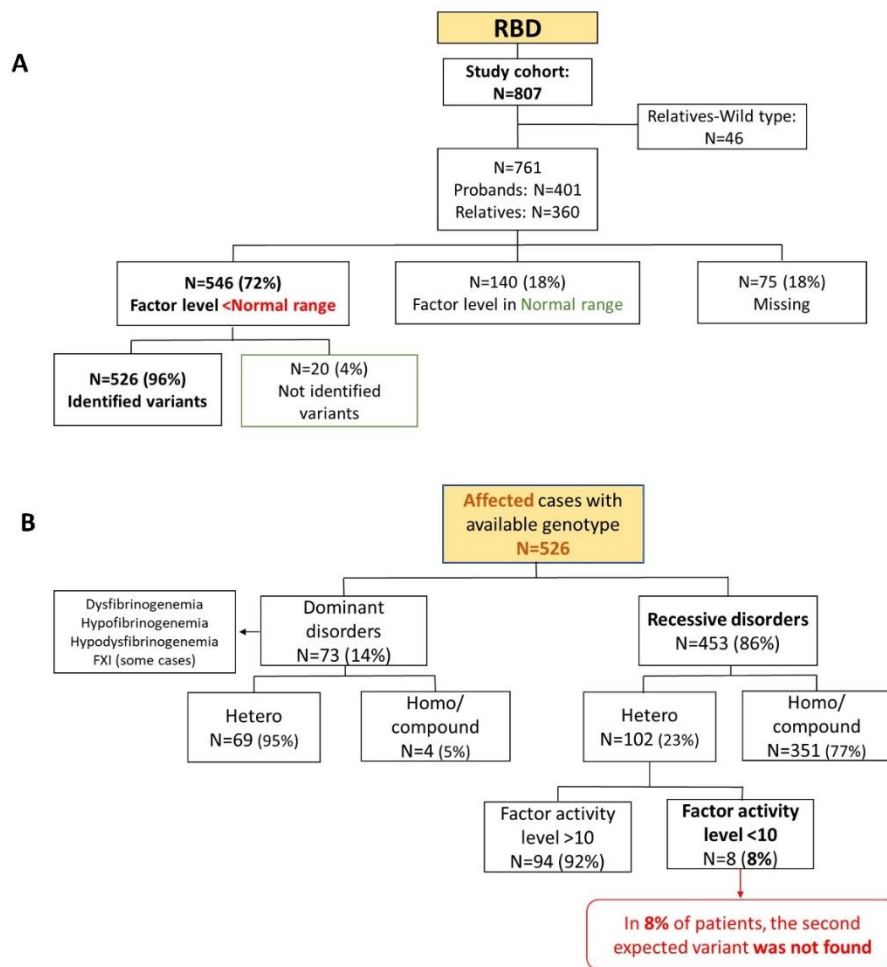


Figure 1. Patients population included in the study. RBD, Rare Bleeding Disorders. **A)** A total of 807 individuals were included in this study of which 526 cases were phenotypically and genotypically characterized. **B)** Following genetic testing, in nearly 8% the second expected variant was not identified.

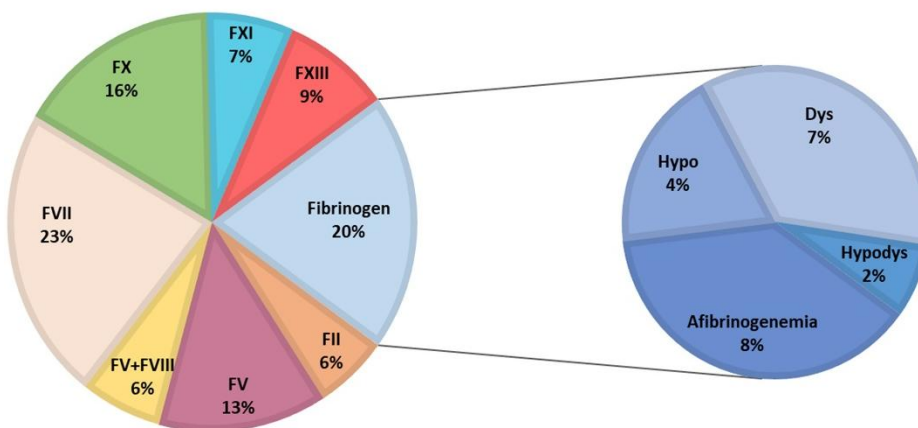


Figure 2. Prevalence of rare bleeding disorders in our cohort. FVII deficiency was the most prevalent disorder in this study, which was followed by fibrinogen deficiencies. Hypo, hypofibrinogenemia; Dys, dysfibrinogenemia; Hypodys, hypodysfibrinogenemia.

Phenotype and genotype characterization

The median and range of coagulation factor activity levels is calculated for each available genotype status as shown in Table 1. Regarding the severity of laboratory phenotypes, patients with FII deficiency were predominantly mildly deficient with a median factor level of 45% (range: 2–66%). Patients with hypofibrinogenemia and dysfibrinogenemia were predominantly mildly deficient, with hypofibrinogenemia cases exhibiting a milder phenotype, with a median factor level of 104 mg/dL (range: 36-140 mg/dL). However, all patients with afibrinogenemia and the majority of those with FV, FVII, FV+FVIII, FX, FXI and FXIII deficiencies were severely deficient (Figure 3). Of the 526 cases, 453 (86%) had an autosomal recessive pattern; 77% (n=351) and 23% (n=102) being homozygote/compound heterozygote and heterozygote, respectively (Figure 1B). Even though the most severe deficiencies were caused by homozygous variants, there were also heterozygous cases of FV, FVII and FX deficiencies who were severely deficient (Table 2). This suggests that the second expected variant in these heterozygous cases was not identified. Severe deficiency was defined as undetectable protein production for fibrinogen disorders and below 10% for the other coagulation deficiencies. Consequently, 8 (8%) heterozygous cases had activity levels below these thresholds and were classified as severe cases. In these cases, individuals were expected to carry more than one mutated allele, but the second expected variant associated with recessive disorders was not identified (Figure 1B). In contrast, 73 (14%) cases exhibited disorders with a dominant pattern, 95% (n=69) being heterozygotes and 5% (n=4) being homozygous or compound heterozygotes (Figure 1B). Regarding hypofibrinogenemic patients, heterozygous cases had higher median factor activity levels (109 mg/dL) compared to homozygous cases (39.5 mg/dL). Thus as expected heterozygous cases were more frequent than homozygous ones (n=18 vs. n=2). There was only one case with dysfibrinogenemia carrying compound heterozygous variants, despite a moderately reduced factor activity levels (52 mg/dL), as summarized in Table 1.

Table 1. Coagulation factor activity levels in different deficiencies stratified by genotypes.

Deficiency	Heterozygous	Homozygous	Compound
Afibrinogenemia (n=40)	n=0	n=36	n=4
		Undetectable	Undetectable
Hypofibrinogenemia (n=20)	n=18	n=2	n=0
	109 (36-140) mg/dl	39.5 (39-40) mg/dl	
Dysfibrinogenemia (n=37)	n=36	n=0	n=1
	52 (0-150) mg/dl		52 mg/dl
Hypodysfibrinogenemia (n=8)	n=7	n=1	n=0
	54 (34-134) mg/dl	20 mg/dl	
FII deficiency (n=31)	n=17	n=11	n=3
	54% (8-66%)	9% (4-61%)	10% (2-26%)
FV deficiency (n=70)	n=21	n=36	n=13
	38% (0-57%)	0% (0-12%)	0% (0-52%)
FV+FVIII deficiency (n=34)	n=0	n=34	n=0
		FV: 11.75% (0-22)% FVIII: 11.5% (4-29)%	
FVII deficiency (n=120)	n=37	n=60	n=23
	28% (0-48%)	0% (0-37%)	0% (0-48%)
FX deficiency (n=85)	n=18	n=58	n=9
	45% (0-64%)	0% (0-30%)	4% (0-32%)
FXI deficiency (n=36)	n=8	n=19	n=9
	39% (23-50%)	0% (0-9%)	0% (0-4%)
FXIII deficiency (n=46)	n=8	n=34	n=4
	50% (48-57%)	0% (0-40%)	0%

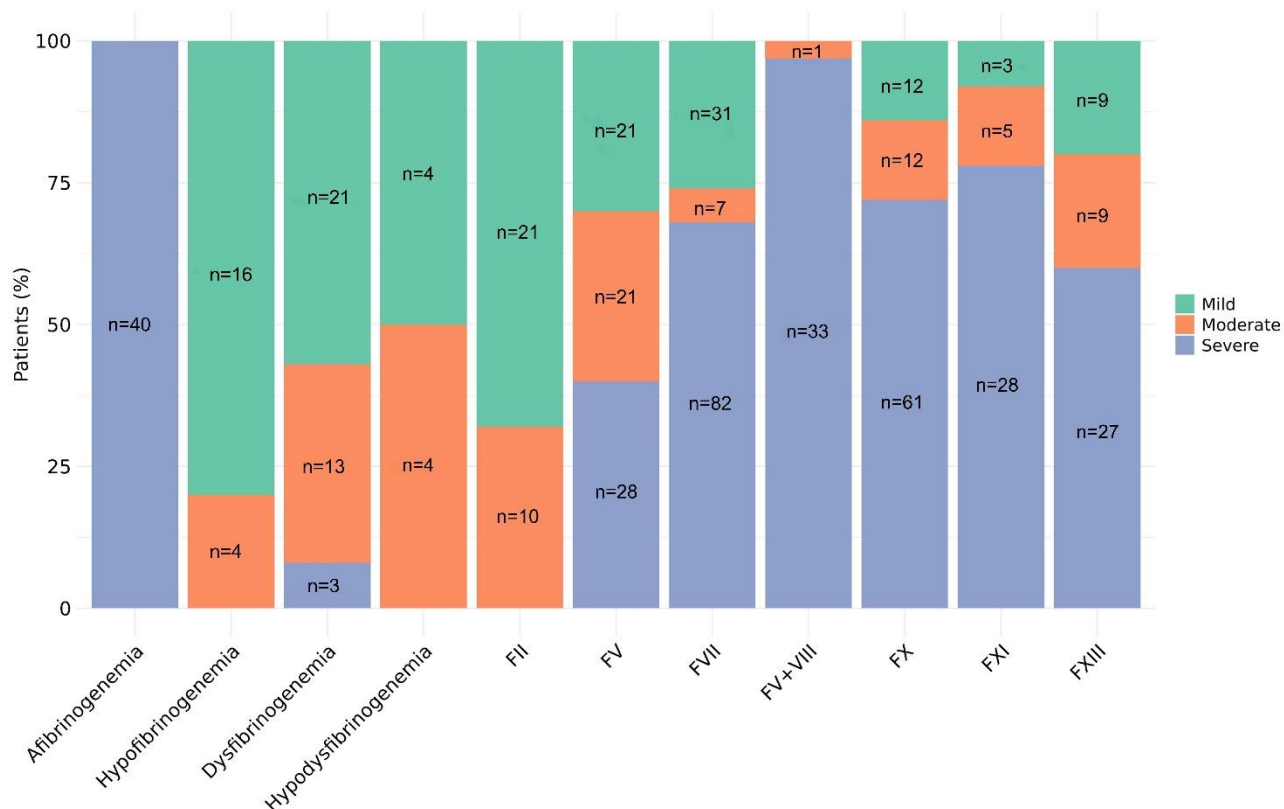


Figure 3. Distribution of the severity of deficiencies based on the factor activity levels. All cases with afibrinogenemia and the majority of those with FV, FVII, FV+VIII, FX, FXI and FXIII deficiencies were severely deficient.

Table 2. Severity of factor deficiencies according to the different genotypes.

Severity of deficiency	Homo	Hetero	Compound
Afibrinogenemia/ Hypofibrinogenemia			
Severe (n=40)	36 (90%) ¹	-	4 (10%) ¹
Moderate (n=4)	2 (50%) ²	2 (50%) ²	-
Mild (n=16)	-	16 (100%) ²	-
Dysfibrinogenemia/ Hypodysfibrinogenemia			
Severe (n=3)	-	3 (100%) ³	-
Moderate (n=17)	1 (6%) ⁴	16 (94%) ⁵	-
Mild (n=25)	-	24 (96%) ⁶	1 (4%) ³
FII			
Severe (n=0)	-	-	-
Moderate (n=10)	7 (70%)	1 (10%)	2 (20%)
Mild (n=21)	4 (19%)	16 (76%)	1 (5%)
FV			
Severe (n=28)	20 (72%)	2 (7%)	6 (21%)
Moderate (n=21)	15 (71%)	1 (5%)	5 (24%)
Mild (n=21)	1 (5%)	18 (86%)	2 (9%)
FVII			
Severe (n=82)	56 (68%)	5 (6%)	21 (26%)
Moderate (n=7)	4 (57%)	3 (43%)	-
Mild (n=31)	1 (3%)	29 (94%)	1 (3%)
FV+FVIII			
Severe (n=33)	33 (100%)	-	-
Moderate (n=1)	1 (100%)	-	-
Mild (n=0)	-	-	-
FX			
Severe (n=61)	56 (92%)	1 (2%)	4 (6%)
Moderate (n=12)	2 (16%)	5 (42%)	5 (42%)
Mild (n=12)	-	12 (100%)	-
FXI			
Severe (n=28)	19 (68%)	-	9 (32%)
Moderate (n=5)	-	5 (100%)	-
Mild (n=3)	-	3 (100%)	-

FXIII			
Severe (n=27)	23 (85%)	-	4 (15%)
Moderate (n=9)	9 (100%)	-	-
Mild (n=9)	1 (11%)	8 (89%)	-

¹Afibrinogenemia; ²Hypofibrinogenemia; ³ Dysfibrinogenemia; ⁴Hypodysfibrinogenemia; ⁵Dysfibrinogenemia, n=13 and Hypodyfibrinogenemia n=3; ⁶Dysfibrinogenemia, n=20 and hypodysfibrinogenemia, n=4.

Mutational spectrum

A total of 590 variants were found, of which 257 were unique (Suppl, Table S2). Missense variants were the most frequent mutation type observed across all deficiencies (57%), except in afibrinogenemia and compound FV+FVIII deficiencies in which nonsense variants were the most common type (52% and 55%, respectively) (Figure 4). Considering the distribution of variants throughout the genes, exons with the highest frequency of variants were exon 5 *FGA* (40%, afibrinogenemia), exon 8 *FGG* (70%, dys/hypofibrinogenemia), exon 10 and 14 *F2* (27%), exon 13 *F5* (22%), exon 9 *F7* (58%), and exon 8 *F10* (37%). All identified variants in FXIII deficiency were located in *F13A1*. To avoid bias caused by different numbers of the family members, the analysis of the mutational spectrum was performed only on variants found in probands (Suppl, Table S2). The most frequent identified variants in probands are listed in Table 3.

Table 3. The most frequent variants identified in probands

Deficiency (probands, n)	cDNA	Protein	Gene/Exon, Intron	Domain	N (%)
Afibrinogenemia (n=37)	c.385C>T	p.Arg129*	<i>FGA</i> / Ex4	-	5 (14%)
	c.139C>T	p.Arg47*	<i>FGB</i> / Ex2	-	4 (11%)
Dysfibrinogenemia (n=28)	c.103C>T	p.Arg35Cys	<i>FGA</i> / Ex2	-	6 (21%)
	c.902G>A	p.Arg301His	<i>FGG</i> / Ex8	-	6 (21%)
FII deficiency (n=14)	-	-	-	-	No recurrent variant
FV (n=50)	c.5189A>G	p.Tyr1730Cys	<i>F5</i> / Ex15	A3	4 (8%)
FVII (n=110)	c.1061C>T	p.Ala354Val	<i>F7</i> / Ex9	Catalytic	10 (9%)
	c.1109G>T	p.Cys370Phe	<i>F7</i> / Ex9	Catalytic	9 (8%)
FV+FVIII (26) LMAN (n=13) MCFD2 (n=13)	c.2T>C	p.Met1Thr	<i>LMAN1</i> /Ex1	-	5 (38%)
	c.149+5G>A	-	<i>MCFD2</i> /Int2	-	8 (62%)
FX (n=55)	c.785G>A	p.Gly262Asp	<i>F10</i> / Ex7	Catalytic	10 (18%)
	c.400G>A	p.Gly134Arg	<i>F10</i> / Ex5	EGF	9 (16%)
FXI (n=30)	c.403G>T	p.Glu135*	<i>F11</i> / Ex5	Apple2	14 (47%)
FXIII (n=34)	c.320G>A	p.Arg77His	<i>F13A1</i> / Ex4	βsandwich	5 (15%)
	c.646G>A	p.Gly216Arg	<i>F13A1</i> / Ex5	Catalytic	4 (12%)

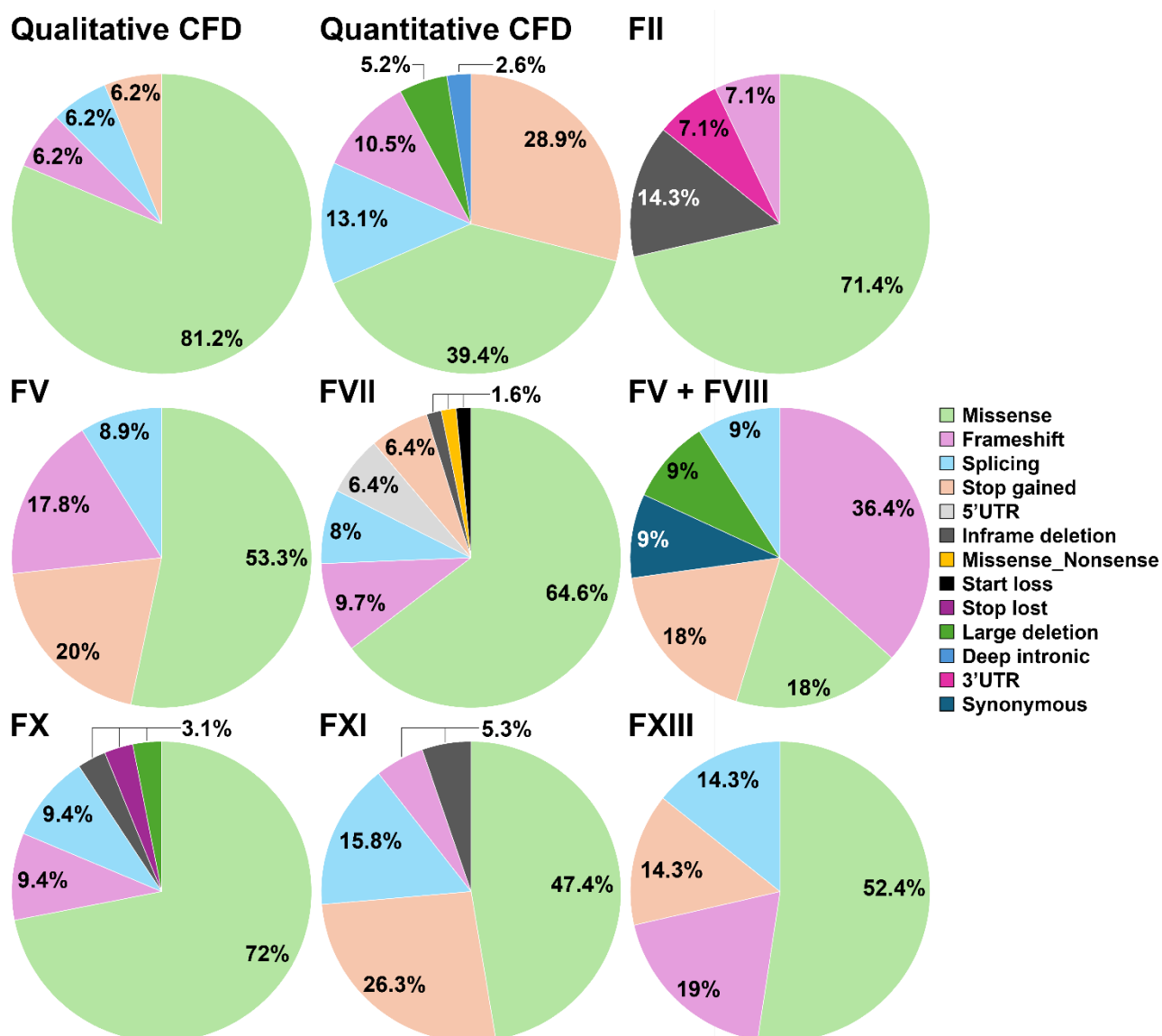


Figure 4. The frequency of different types of variants found in patients with the rare bleeding disorders. Except for afibrinogenemia and compound FV+FVIII deficiencies, where nonsense variants were the most common, missense variants were the most prevalent type in the remaining deficiencies. Quantitative congenital fibrinogen disorders (CFDs) include afibrinogenemia and hypofibrinogenemia; qualitative CFDs include dysfibrinogenemia and hypodysfibrinogenemia.

Variants distribution in domains

The domain distribution for all identified variants is reported in the supplementary Table S2. In brief, most variants affected the catalytic B chain in FII (69%), A3 (29%) and B (20%) domains in FV, and the serine protease domain in FVII (72%). For FX deficiency, 54% were in the catalytic domain; in FXI deficiency, variants were clustered in the serine protease (33%) and Apple4 (28%) domains, and

in FXIII deficiency, 72% affected the catalytic domain. Overall, 82 of 172 (48%) distinct coding-region variants were located in catalytic domains.

Pathogenicity and novelty of variants

Of the 257 distinct identified variants, 230 (89%) were previously reported to be associated with coagulation factor deficiencies, while 28 (11%) variants were novel (Figure 5A). According to the CADD score prediction tool, 86% of all the 257 identified variants were predicted to be pathogenic (i.e. CADD > 20), 14% were benign. Regarding the 28 novel variants, 21 (75%) were predicted to be pathogenic and 7 (25%) as benign. To compare the pathogenicity of reported and novel variants, we performed a CADD distribution for both groups (Figure 5B). The results suggested that the pathogenicity of novel variants was almost the same as those previously reported. Among the 140 missense variants predicted pathogenic by the REVEL score, 130 (92%) were also predicted pathogenic by the CADD score. Following searching the pathogenic variants in gnomAD, p.Thr577Met in *F13A1*, p.Phe301Leu and p.Glu135* in *F11* and p.Asp473Asn in *FGA* were found with the highest frequency, although still with low MAF in the general population (0.002, 0.0006, 0.0005, 0.0005, respectively).

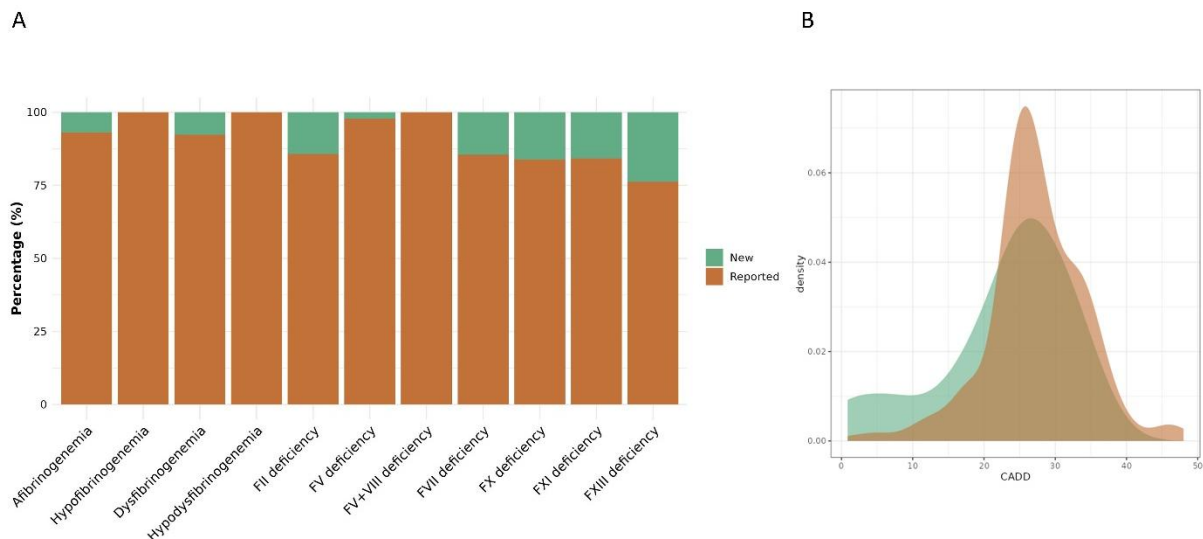


Figure 5. Rate of novel variants and assessment of their pathogenicity. A) A total of 28 novel variants were found and the proportion of reported and novel variants in the study population are shown for each deficiency. B) CADD distribution of reported and novel variants identified. The pathogenicity of novel variants was almost the same as those previously reported. A CADD score >20 is considered pathogenic.

Genotype phenotype correlation

A total of 257 distinct variants were evaluated across all cases to investigate any potential association between the severity of the disease and the presence of these variants (Suppl, Figure S2). In afibrinogenemia, the homozygous *FGA*, p.Arg129* and p.Arg168* variants were the most frequent, found in 5 and 4 cases. In dysfibrinogenemia, Arg301 residue variants were commonly observed, with *FGG*, p.Arg301His associated with higher median factor activity than *FGG*, p.Arg301Cys (67 vs. 45 mg/dL). Unlike other deficiencies, all identified variants in FII deficiency were linked to mild or moderate deficiency (>1%), although the homozygous p.Arg42Trp variant might be associated with severe deficiency. In FV deficiency, the p.Tyr1730Cys and p.Ser955Alafs*4 variants were more frequent (n=4) in severe cases, potentially contributing to low factor activity levels. In FVII deficiency, the homozygous p.Cys370Phe variant was identified in 9 cases, all of whom had severe factor levels deficiency. For FX deficiency, the homozygous p.Gly262Asp and p.Gly134Arg variants were found in 14 and 8 cases, respectively, indicating a strong association with severe deficiency. In FXI deficiency, the homozygous p.Glu135* variant (n=8), as well as the homozygous p.Arg77His (n=5) and c.1459+1G>A (n=5) variants in FXIII deficiency, were predominantly observed in severely deficient cases, suggesting a link to severe deficiency (Suppl, Figure S2).

Discussion

The rarity of RBDs has led to limited studies on their genetics, diagnosis, and management. Therefore, conducting studies on large cohorts is essential to understand more on RBDs. Previous studies on RBDs focused on small cohorts and single-factor deficiencies, while this study, the largest to date, analyzes the genetics and phenotype-genotype characteristics of 526 cases, with equal sex representation. The evaluation of the worldwide distribution of the RBDs, based on surveys from the WFH (<http://www.wfh.org/>) and the EN-RBD (<http://www.rbdd.eu/>), indicated that FVII and FXI deficiencies are the most prevalent, while FII and FV+FVIII deficiencies were the less frequent of the RBDs. In our cohort, the most common RBD was FVII deficiency (23%), followed by fibrinogen deficiencies (20%). This may be due to including data from our previous project on patients with fibrinogen deficiencies (PRO-RBDD) [21]. In addition, cases with FII and compound FV+FVIII deficiencies had a higher frequency (6%) compared to the worldwide reports ($\approx$ 2-3%) [22, 23].

In our study, more than half of the cases had severe factor deficiency (58%), except for cases with hypofibrinogenemia, dysfibrinogenemia, hypodysfibrinogenemia, and FII deficiencies, who had mild to moderate deficiency. Severe FII deficiency appears to be incompatible with life [24, 25]. All cases

with FII deficiency in our study were mild to moderate, likely contributing to the higher observed case count.

The high frequency of nonsense variants in afibrinogenemia and FV+FVIII deficiency, and missense variants in the other RBDs, suggesting a quantitative and dysfunctional nature of these deficiencies, respectively. These findings are consistent with previous reports [24, 26]. Since the catalytic domain was the most affected one in our study, its extended lengths may contribute to a higher incidence of embedded variants [27]. The distribution of observed variants in a previous report highlighted the catalytic domain as the most affected region, encompassing 57% of variants in FII, 32% in FX, and 55% in FXIII deficiencies [2]. Our findings further support this trend, showing an even higher proportion of affected catalytic domains in these deficiencies. The catalytic domain plays a crucial role in the function of these coagulation factors, and its high variant frequency underscores its vulnerability to pathogenic variants. The majority of our nonsense variants in FV deficiency were identified in the exons encoding B and A3 domains, while most missense variants affected the A2 and C2 domains, with none found in the B domain that is in agreement with a previous report [28].

A large number of homozygous or compound heterozygous cases were severely deficient, nonetheless, there were few homozygous cases with mild or moderate deficiency (Table 2). As an example, although hypofibrinogenemia is a dominant disorder, there were two homozygous cases with p.Arg129Pro variant who were moderately deficient. This may be explained by the fact that this variant does not seem to affect fibrinogen synthesis and secretion [29, 30].

The *FGA*, p.Arg168* variant has previously been identified in five cases of congenital afibrinogenemia, all presenting with very low to undetectable fibrinogen levels and thus suggesting that loss-of-function is the underlying disease mechanism [21, 31, 32]. The *FGA*, p.Arg129* was the other identified variant in afibrinogenemia associated with severe reduced fibrinogen levels. Although this variant was previously reported as likely pathogenic [33], its CADD score of 36 and its association with severe deficiency meet our criteria for a classification as pathogenic. The *FGG*, Arg301 residue is a hotspot in dysfibrinogenemia, with 38% of our identified variants being located at this site, in agreement with our previous study, which reported 34% [21]. Among the associated variants with FII deficiency, the homozygous variant p.Arg42Trp was notably present in cases with factor levels below 10%. This variant has been reported only once as a variant of uncertain significance in ClinVar [33]. However, given its CADD score of 26 and a MAF of 0.000002, our findings suggest a pathogenic nature for this variant. The p.Cys370Phe was found in cases with severe FVII deficiency that aligns with previous reports [34]. As noted in previous reports, the p.Gly262Asp

variant was associated with severe deficiency, and in the current study it was identified in 14 severe FX deficiency cases [35]. Consistent with previous studies, p.Glu135* was prevalent and strongly associated with FXI deficiency, being more prevalent in Ashkenazi Jews and Italians [36]. Among *F13* variants, p.Arg77His was also prominently found in severe cases, aligning with a previous study [37]. Collectively, these spots can be recommended as a candidate for mutational screening analysis. In a study by Zhang et al., variants in *MCFD2* were associated with lower levels of FV and FVIII as compared to *LMAN1* [38]. In contrast, our findings show that variants in *MCFD2* and *LMAN1* genes are associated with similar low median factor activity levels. Specifically, *MCFD2* variants with 12% FV and 10% FVIII, and *LMAN1* variants with 12% FV and 15% FVIII. One possible reason for the discrepancy could be differences in study populations, such as genetic background and ethnicity, which may influence the observed effects of mutations on factor levels. Additionally, variability in the specific mutations within *MCFD2* and *LMAN1* could contribute to differences in clinical outcomes, as not all variants in these genes may exert the same impact on protein function.

In 8% of heterozygous cases who had severe deficiency, a second pathogenic variant may have been missed, potentially due to noncoding variants, regulatory region changes, large deletions/duplications, or genomic rearrangements [39]. Therefore, while PCR-based Sanger sequencing has been the standard in many diagnostic settings, next-generation sequencing approaches such as whole exome or whole genome now offer more comprehensive and efficient strategies for genotyping RBDs and are increasingly being integrated into clinical diagnostics. Additionally, post-translational modifications such as methylation, which can inhibit gene transcription, may play a role in the disease severity [40]. Future studies should apply alternative approaches for these patients, including multiplex ligation-dependent probe amplification (MLPA) for the identification of copy number variations [41], long read sequencing for resolving some of the more complex structural variants [42] and long-range PCR for the detection of exon deletion [43]. The role of unknown genes that modulate coagulation factor plasma levels cannot be overlooked. For example, FV level was reported to be modulated by the *PLXDC2* locus [44]. Using whole genome sequencing, several regions associated with plasma fibrinogen levels were recently identified [45]. In addition, genome-wide association studies (GWAS) have highlighted the role of multiple genetic factors in bleeding disorders like hemophilia and von Willebrand disease [46]. These findings could provide new insights by uncovering additional genetic loci linked to RBDs. However, while GWAS can identify potential genetic modifiers, its direct application to genetic diagnosis in RBDs is still limited. The variants identified in such studies may not always be directly actionable, and their clinical relevance would require further validation in larger and diverse populations.

Identifying novel variants (11%), highlighting both the genetic heterogeneity of RBDs and the presence of many previously unreported variants. While 89% of variants have already been identified, the remaining variants may include previously undetected changes such as structural variants, regulatory alterations, or non-coding variants that could offer deeper insights into disease mechanisms. As genetic studies on RBD patients expand, the rising number of novel variants poses challenges in understanding their roles in disease mechanisms, especially given the lack of functional testing for many reported variants. To investigate them, large databases like gnomAD can filter out common variants, and in silico tools can assess their pathogenic effects. Studying family members is also crucial for understanding variant pathogenicity and inheritance patterns [47]. Our result suggested that the pathogenicity of novel variants is similar to that of previously reported.

Despite advances in genetic characterization over the past two decades, 5–10% of patients with severe RBDs still lack identifiable genetic defects [22]. In our cohort, in 4% of cases no causative variant was identified of which 20% had severe deficiency.

This study has several limitations. First limitation is the lack of clinical data to correlate bleeding severity with factor activity levels. Second, there was a higher representation of fibrinogen deficiency cases, likely reflecting our center's focus and potentially skewing results in comparison to global RBD trends. Third, all patients with afibrinogenemia and most individuals with FV, FVII, FVIII, FXI, and FXIII deficiencies exhibited severe deficiency, this may be due to the inclusion of patients with severe symptoms. Finally, copy number variant analysis was not performed for patients with a suspected missing second variant. The influence of ethnicity should not be overlooked, as it could represent another limitation of this study.

Conclusions

This study investigated the mutational spectrum of RBDs using one of the largest cohorts collected over 19 years. This large cohort has allowed us to collect detailed information on 11 different genes responsible for RBDs. In all deficiencies, missense was the most prevalent type of variant except for afibrinogenemia and compound FV and FVIII and most of the identified variants affect the catalytic domain. With such a large sample, our results examined variants with the most severe reduced factor activity levels in each coagulation disorder to understand the genotype/phenotype correlation of RBDs. In our cohort, no causative variant was identified in 4% of cases, and in nearly 8% of patients with severe deficiency, a second expected pathogenic variant remained undetected. Further investigation is required for these patients in order to understand the genetic mechanism underlying the deficiency.

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Supplementary

Table S1. Severity classification based on factor activity levels as proposed by the International Society on Thrombosis and Haemostasis (ISTH).

Deficiency	Laboratory phenotype		
	Coagulant activity		
	Severe	Moderate	Mild
Fibrinogen	Undetectable	0.1–1 g/L	>1 g/L
Prothrombin	Undetectable	≤10%	>10%
Factor V	Undetectable	<10%	≥10%
Combined FV and FVIII	<20%	20%–40%	>40%
Factor VII	<10%	10%–20%	>20%
Factor X	<10%	10%–40%	>40%
Factor XIII	<10%	10%–30%	≥30%

Table S2. Characteristics of identified distinct variants.

Deficiency	Gene	c.DNA	Protein	Type of variants	Exon	Intron	Domain	Reported/ New	CADD	REVEL
Afibrinogenemia	FGA	c.1072G>T	p.Gly358*	Stop gained	5		N.A	New	32	N.A
Afibrinogenemia	FGA	c.117delT	p.Val40Trpfs*31	Frameshift	2		N.A	Reported	17.3	N.A
Afibrinogenemia	FGA	c.1217del	p.Asn406Thrfs*15	Frameshift	5		N.A	New	15.73	N.A
Afibrinogenemia	FGB	c.139C>T	p.Arg47*	Stop gained	2		N.A	Reported	37	N.A
Afibrinogenemia	FGA	c.15kbDel	c.15kbDel	large deletion			N.A	Reported		N.A
Afibrinogenemia	FGA	c.196dup	p.Ser66Phefs*10	Frameshift	3		N.A	Reported	35	N.A
Afibrinogenemia	FGA	c.24C>A	p.Cys8*	Stop gained	1		N.A	Reported	36	N.A
Afibrinogenemia	FGA	c.356C>G	p.Ser119*	Stop gained	3		N.A	Reported	22.9	N.A
Afibrinogenemia	FGA	c.385C>T	p.Arg129*	Stop gained	4		N.A	Reported	36	N.A
Afibrinogenemia	FGA	c.4.1kbdel	c.4.1kbdel	large deletion			N.A	Reported		N.A
Afibrinogenemia	FGA	c.484C>T	p.Gln162*	Stop gained	4		N.A	Reported	36	N.A
Afibrinogenemia	FGA	c.502C>T	p.Arg168*	Stop gained	4		N.A	Reported	35	N.A
Afibrinogenemia	FGA	c.54+1G>A	c.54+1G>A	Splicing		1	N.A	Reported	34	N.A
Afibrinogenemia	FGA	c.598C>T	p.Gln200*	Stop gained	5		N.A	Reported	36	N.A
Afibrinogenemia	FGA	c.609_610insTGA	p.Leu204*	Stop gained	5		N.A	Reported	33	N.A
Afibrinogenemia	FGA	c.743G>A	p.Trp248*	Stop gained	5		N.A	Reported	29.9	N.A
Afibrinogenemia	FGA	c.945delT	p.Gly316Glufs*105	Frameshift	5		N.A	Reported	4.364	N.A
Afibrinogenemia	FGA	c.94G>T	p.Gly32*	Stop gained	2		N.A	Reported	37	N.A
Afibrinogenemia	FGB	c.1148T>G	p.Leu353Arg	Missense	7		N.A	Reported	28.2	0.96799
Afibrinogenemia	FGB	c.1220C>A	p.Thr407Lys	Missense	7		N.A	Reported	26.3	0.99762
Afibrinogenemia	FGB	c.1244+1G>T	c.1244+1G>T	Splicing		7	N.A	Reported	34	N.A
Afibrinogenemia	FGB	c.1288G>C	p.Gly430Arg	Missense	8		N.A	Reported	31	0.99157
Afibrinogenemia	FGB	c.1399T>G	p.Trp467Gly	Missense	8		N.A	Reported	32	0.98489
Afibrinogenemia	FGB	c.139C>T	p.Arg47*	Stop gained	2		N.A	Reported	36	N.A
Afibrinogenemia	FGB	c.605T>A	p.Leu202Gln	Missense	4		N.A	Reported	32	0.93464

Afibrinogenemia	FGB	c.958+13C>T	c.958+13C>T	Splicing		6	N.A	Reported	1.228	N.A
Afibrinogenemia	FGG	c.123+1G>A	c.123+1G>A	Splicing		2	N.A	Reported	34	N.A
Afibrinogenemia	FGG	c.667-320A>T	c.667-320A>T	Deep intronic		6	N.A	Reported	13.52	N.A
Afibrinogenemia	FGG	c.78+5G>A	c.78+5G>A	Splicing		1	N.A	Reported	24.5	N.A
Hypofibrinogenemia	FGA	c.112A>G	p.Arg38Gly	Missense	2		N.A	Reported	27.3	0.83582
Hypofibrinogenemia	FGA	c.209T>G	p.Met70Arg	Missense	3		N.A	Reported	27.3	0.94728
Hypofibrinogenemia	FGA	c.386G>C	p.Arg129pro	Missense	4		N.A	Reported	18.8	0.77847
Hypofibrinogenemia	FGA	c.552C>G	p.Cys184Trp	Missense	5		N.A	Reported	23.3	0.98489
Hypofibrinogenemia	FGB	c.1391G>A	p.Gly464Asp	Missense	8		N.A	Reported	28.9	0.96614
Hypofibrinogenemia	FGB	c.283T>C	p.Cys95Arg	Missense	2		N.A	Reported	26.8	0.99803
Hypofibrinogenemia	FGG	c.1024G>A	p.Asp342Asn	Missense	8		N.A	Reported	28.7	0.94728
Hypofibrinogenemia	FGG	c.1112A>G	p.Asn371Ser	Missense	8		N.A	Reported	26.3	0.98489
Hypofibrinogenemia	FGG	c.1201C>T	p.Arg401Trp	Missense	9		N.A	Reported	26.2	0
Dysfibrinogenemia	FGA	c.100G>A	p.Val34Met	Missense	2		N.A	Reported	23.2	0.29158
Dysfibrinogenemia	FGA	c.103C>T	p.Arg35Cys	Missense	2		N.A	Reported	32	0.74588
Dysfibrinogenemia	FGA	c.104G>A	p.Arg35His	Missense	2		N.A	Reported	28.1	0.6823
Dysfibrinogenemia	FGA	c.1410_1411insT	p.Gly471Trpfs*3	Frameshift	5		N.A	Reported	23	N.A
Dysfibrinogenemia	FGA	c.1417G>A	p.Asp473Asn	Missense	5		N.A	Reported	23.9	0.55752
Dysfibrinogenemia	FGB	c.1244+1G>A	c.1244+1G>A	Splicing		7	N.A	Reported	34	N.A
Dysfibrinogenemia	FGB	c.643G>A	p.Asp215Asn	Missense	4		N.A	Reported	23.7	0.67691
Dysfibrinogenemia	FGG	c.1054T>C	p.Cys326Arg	Missense	8		N.A	New	31	0.99024
Dysfibrinogenemia	FGG	c.1067A>T	p.Asp356Val	Missense	8		N.A	Reported	25.1	0.96799
Dysfibrinogenemia	FGG	c.1073C>G	p.Ser358Cys	Missense	8		N.A	Reported	29.8	0.99783
Dysfibrinogenemia	FGG	c.1146A>T	p.Lys382Asn	Missense	9		N.A	Reported	20.9	0.5534
Dysfibrinogenemia	FGG	c.901C>T	p.Arg301Cys	Missense	8		N.A	Reported	32	0.97398
Dysfibrinogenemia	FGG	c.902G>A	p.Arg301His	Missense	8		N.A	Reported	29.4	0.94616
Hypodysfibrinogenemia	FGA	c.191G>T	p.Cys64Phe	Missense	3		N.A	Reported	25.9	0.98041
Hypodysfibrinogenemia	FGA	c.885G>A	p.Trp295*	Stop gained	5		N.A	Reported	33	N.A
Hypodysfibrinogenemia	FGG	c.1151C>G	p.Ser384Cys	Missense	9		N.A	Reported	25.3	0.75211
FII deficiency	F2	c.*80T>C	c.*80T>C	3'UTR			N.A	New	16.68	N.A
FII deficiency	F2	c.1029_1034del	p.Lys344_Lys345del	Inframe Deletion	9		Catalytic Bchain	Reported	18.33	N.A
FII deficiency	F2	c.1117G>A	p.Gly373Ser	Missense	9		Catalytic Bchain	Reported	25.3	0.91583
FII deficiency	F2	c.1189A>C	p.Ser397Arg	Missense	10		Catalytic Bchain	Reported	28.6	0.98921
FII deficiency	F2	c.1206_1226del	p.Thr403_Leu409del	Inframe Deletion	10		Catalytic Bchain	New	21.1	N.A
FII deficiency	F2	c.124C>T	p.Arg42Trp	Missense	2		Gla	Reported	26.2	0.95396
FII deficiency	F2	c.1274G>A	p.Arg425His	Missense	10		Catalytic Bchain	Reported	32	0.97173
FII deficiency	F2	c.128G>A	p.Arg43Gln	Missense	2		Gla	Reported	25.5	0.93108
FII deficiency	F2	c.1514G>A	p.Gly505Asp	Missense	12		Catalytic Bchain	Reported	29.8	0.99822
FII deficiency	F2	c.1741C>T	p.Arg581Cys	Missense	14		Catalytic Bchain	Reported	24.8	0.942
FII deficiency	F2	c.1814A>G	p.His605Arg	Missense	14		Catalytic Bchain	Reported	24.1	0.95433
FII deficiency	F2	c.1819_1820del	p.Phe607Profs*11	Frameshift	14		Catalytic Bchain	Reported	33	N.A
FII deficiency	F2	c.481G>T	p.Asp161Tyr	Missense	6		LNK2	Reported	24.4	0.87576
FII deficiency	F2	c.787C>T	p.Arg263Cys	Missense	7		LNK3	Reported	26.1	0.99545
FV deficiency	F5	c.1118+6T>C	c.1118+6T>C	Splicing		7	N.A	Reported	21.8	N.A
FV deficiency	F5	c.1309A>G	p.Asn437Asp	Missense	9		A2	Reported	28.3	0.92087
FV deficiency	F5	c.1321C>T	p.Arg441Cys	Missense	9		A2	Reported	32	0.9789
FV deficiency	F5	c.1430C>G	p.Pro477Arg	Missense	10		A2	Reported	28.3	0.96836

FV deficiency	F5	c.1498T>G	p.Cys500Gly	Missense	10		A2	Reported	29	0.9999
FV deficiency	F5	c.158+1G>A	c.158+1G>A	Splicing		1	N.A	Reported	33	N.A
FV deficiency	F5	c.1652T>C	p.Phe551Ser	Missense	11		A2	Reported	32	0.98886
FV deficiency	F5	c.1883G>A	p.Gly628Glu	Missense	12		A2	Reported	25.9	0.98079
FV deficiency	F5	c.2218C>T	p.Arg740*	Stop gained	13		B	Reported	37	N.A
FV deficiency	F5	c.2574_2575del	p.Gln860Argfs*4	Frameshift	13		B	Reported	18.81	N.A
FV deficiency	F5	c.2841G>A	p.Trp947*	Stop gained	13		B	Reported	39	N.A
FV deficiency	F5	c.2862del	p.Ser955Alafs*4	Frameshift	13		B	Reported	22.1	N.A
FV deficiency	F5	c.3088C>T	p.Arg1030*	Stop gained	13		B	Reported	40	N.A
FV deficiency	F5	c.320A>G	p.Asp107Gly	Missense	3		A1	Reported	25.5	0.75279
FV deficiency	F5	c.3296del	p.Asp1099Alafs*72	Frameshift	13		B	Reported	17.98	N.A
FV deficiency	F5	c.333C>A	p.Ser111Arg	Missense	3		A1	Reported	25.6	0.99221
FV deficiency	F5	c.3481C>T	p.Arg1161*	Stop gained	13		B	Reported	35	N.A
FV deficiency	F5	c.3799del	p.Leu1267Phefs*21	Frameshift	13		B	Reported	18.2	N.A
FV deficiency	F5	c.44G>A	p.Gly15Asp	Missense	1		A1	Reported	24.8	0.84291
FV deficiency	F5	c.4650C>G	p.Tyr1550*	Stop gained	13		A3	Reported	38	N.A
FV deficiency	F5	c.4900C>T	p.Arg1634*	Stop gained	14		A3	Reported	39	N.A
FV deficiency	F5	c.5005T>C	p.Ser1669Pro	Missense	15		A3	Reported	27.8	0.97814
FV deficiency	F5	c.5093del	p.Asn1698Metfs*37	Frameshift	15		A3	Reported	33	N.A
FV deficiency	F5	c.5127_5128insA	p.Trp1710Metfs*5	Frameshift	15		A3	Reported	33	0
FV deficiency	F5	c.5141_5142insTTTC	p.Glu1714Aspfs*46	Frameshift	15		A3	New	21.1	N.A
FV deficiency	F5	c.5189A>G	p.Tyr1730Cys	Missense	15		A3	Reported	32	0.99695
FV deficiency	F5	c.5215G>T	p.Asp1739Tyr	Missense	16		A3	Reported	29	0.9617
FV deficiency	F5	c.5521G>A	p.Val1841Met	Missense	17		A3	Reported	25.7	0.88867
FV deficiency	F5	c.5630C>A	p.Ser1877*	Stop gained	18		A3	Reported	47	N.A
FV deficiency	F5	c.5646G>A	p.Trp1882*	Stop gained	18		A3	Reported	45	N.A
FV deficiency	F5	c.5683G>C	p.Gly1895Arg	Missense	18		A3	Reported	27	0.95912
FV deficiency	F5	c.5765A>C	p.Gln1922Pro	Missense	19		C1	Reported	26.8	0.9691
FV deficiency	F5	c.5788+3A>T	c.5788+3A>T	Splicing		19	N.A	Reported	23.4	N.A
FV deficiency	F5	c.5789G>A	p.Gly1930Asp	Missense	20		C1	Reported	10.2	0.62729
FV deficiency	F5	c.5807T>A	p.Leu1936*	Stop gained	20		C1	Reported	48	N.A
FV deficiency	F5	c.5993G>T	p.Ser1998Ile	Missense	21		C1	Reported	26	0.98563
FV deficiency	F5	c.6048+1G>A	c.6048+1G>A	Splicing		21	N.A	Reported	34	N.A
FV deficiency	F5	c.6122_6123insAACAG	p.Arg2042Thrfs*24	Frameshift	22		C1	Reported	34	N.A
FV deficiency	F5	c.6182G>A	p.Cys2061Tyr	Missense	22		C1	Reported	26.6	0.95728
FV deficiency	F5	c.6197G>A	p.Cys2066Tyr	Missense	23		C2	Reported	25.3	0.94691
FV deficiency	F5	c.6293C>G	p.Pro2098Arg	Missense	23		C2	Reported	27.6	0.96133
FV deficiency	F5	c.6304C>T	p.Arg2102Cys	Missense	23		C2	Reported	31	0.9899
FV deficiency	F5	c.6305G>A	p.Arg2102His	Missense	23		C2	Reported	28	0.97965
FV deficiency	F5	c.6492G>C	p.Trp2164Cys	Missense	24		C2	Reported	29.9	0.98304
FV deficiency	F5	c.6649G>A	p.Glu2217Lys	Missense	25		C2	Reported	27.4	0.99284
FV+VIII deficiency	LMAN1	c.89dup	p.Asp31Argfs*72	Frameshift	1		N.A	Reported	27.4	N.A
FV+VIII deficiency	LMAN1	c.2T>C	p.Met1Thr	Missense	1		N.A	Reported	29.2	0.79033
FV+VIII deficiency	LMAN1	c.340G>T	p.Gly114*	Stop gained	2		L-type lectin-like	Reported	38	N.A
FV+VIII deficiency	LMAN1	c.813_822+62del	p.Lys272fs	Frameshift	7	7	N.A	Reported	28.2	N.A
FV+VIII deficiency	LMAN1	c.822G>A	p.Pro274Pro	Synonymous	7		N.A	Reported	24.1	N.A
FV+VIII deficiency	LMAN1	c.904A>T	p.Lys302*	Stop gained	8		N.A	Reported	41	N.A
FV+VIII deficiency	LMAN1	Deletion_Ex1	Deletion_Ex1	large deletion			N.A	Reported		N.A
FV+VIII deficiency	MCFD2	c.103del	p.Asn35Glnfs*22	Frameshift	2		N.A	Reported	24.1	N.A
FV+VIII deficiency	MCFD2	c.149+5G>A	c.149+5G>A	Splicing		2	N.A	Reported	25.9	N.A
FV+VIII deficiency	MCFD2	c.211_245del	p.Glu71*	Frameshift	3		EFhand 1	Reported	35	N.A
FV+VIII deficiency	MCFD2	c.365A>T	p.Asp122Val	Missense	4		EFhand 2	Reported	29.8	0.99803

FVII deficiency	F7	c.-55C>T	c.-55C>T	5'UTR			N.A	Reported	12.63	N.A
FVII deficiency	F7	c.-61T>G	c.-61T>G	5'UTR			N.A	Reported	17.53	N.A
FVII deficiency	F7	c.-65G>C	c.-65G>C	5'UTR			N.A	Reported	14.18	N.A
FVII deficiency	F7	c.-97del	c.-97del	5'UTR			N.A	New	1	N.A
FVII deficiency	F7	c.1009C>T	p.Arg337Cys	Missense	9		Catalytic	Reported	24.1	0.88013
FVII deficiency	F7	c.1026C>G	p.Ser342Arg	Missense	9		Catalytic	Reported	17.08	0.90939
FVII deficiency	F7	c.1027G>A	p.Gly343Ser	Missense	9		Catalytic	Reported	26.6	0.99931
FVII deficiency	F7	c.1031G>A	p.Trp344*	Stop gained	9		Catalytic	Reported	45	N.A
FVII deficiency	F7	c.1061C>T	p.Ala354Val	Missense	9		Catalytic	Reported	23.5	0.65913
FVII deficiency	F7	c.[1061C>T;1391del]	p.Ala354Val_p.Pro464Hisfs*32	Missense_Nonsense	9		Catalytic	Reported		N.A
FVII deficiency	F7	c.1074G>A	p.Met358Ile	Missense	9		Catalytic	Reported	24	0.85406
FVII deficiency	F7	c.1087C>A	p.Pro363Thr	Missense	9		Catalytic	Reported	24.5	0.94162
FVII deficiency	F7	c.1091G>A	p.Arg364Gln	Missense	9		Catalytic	Reported	25.7	0.88013
FVII deficiency	F7	c.1091G>T	p.Arg364Leu	Missense	9		Catalytic	New	25.7	0.87035
FVII deficiency	F7	c.10C>T	p.Gln4*	Stop gained	1		Signal peptide	Reported	33	N.A
FVII deficiency	F7	c.1109G>T	p.Cys370Phe	Missense	9		Catalytic	Reported	27.9	0.96688
FVII deficiency	F7	c.1151C>T	p.Thr384Met	Missense	9		Catalytic	Reported	23.8	0.93854
FVII deficiency	F7	c.1169C>T	p.Ala390Val	Missense	9		Catalytic	Novel	24.5	0.849
FVII deficiency	F7	c.1171G>A	p.Gly391Ser	Missense	9		Catalytic	Reported	24.8	0.96207
FVII deficiency	F7	c.1208del	p.Asp403Alafs*16	Frameshift	9		Catalytic	Reported	34	N.A
FVII deficiency	F7	c.1223A>G	p.His408Arg	Missense	9		Catalytic	Reported	23	0.90101
FVII deficiency	F7	c.1256C>T	p.Thr419Met	Missense	9		Catalytic	Reported	23.9	0.88157
FVII deficiency	F7	c.1268G>T	p.Ser423Ile	Missense	9		Catalytic	Reported	26.5	0.96947
FVII deficiency	F7	c.1271_1272delinsTT	p.Trp424Phe	Missense	9		Catalytic	Reported	27.4	N.A
FVII deficiency	F7	c.1272G>A	p.Trp424*	Stop gained	9		Catalytic	Reported	46	N.A
FVII deficiency	F7	c.1283G>T	p.Cys428Phe	Missense	9		Catalytic	New	25.9	0.92294
FVII deficiency	F7	c.1285G>A	p.Ala429Thr	Missense	9		Catalytic	Reported	24.7	0.92253
FVII deficiency	F7	c.131-11G>A	c.131-11G>A	Splicing		2	N.A	Reported	5.809	N.A
FVII deficiency	F7	c.1384C>T	p.Arg462*	Stop gained	9		Catalytic	Reported	35	N.A
FVII deficiency	F7	c.1391del	p.Pro464Hisfs*32	Frameshift	9		Catalytic	Reported	22.8	N.A
FVII deficiency	F7	c.204del	p.Phe69Serfs*59	Frameshift	3		Gla	Reported	25.1	N.A
FVII deficiency	F7	c.218T>A	p.Leu73Gln	Missense	3		Gla	Reported	31	0.97173
FVII deficiency	F7	c.230G>A	p.Cys77Tyr	Missense	3		Gla	New	30	0.95175
FVII deficiency	F7	c.244T>C	p.Cys82Arg	Missense	3		Gla	Reported	27.9	0.96243
FVII deficiency	F7	c.[244T>C;751G>C]	p.Cys82Arg_p.Ala251Pro	Missense_Missense	3_8		Gla_Catalytic	Reported		N.A
FVII deficiency	F7	c.247T>C	p.Ser83Pro	Missense	3		Gla	Reported	25.5	0.93306
FVII deficiency	F7	c.262C>G	p.Arg88Gly	Missense	3		Gla	Reported	23.1	0.90721
FVII deficiency	F7	c.291+1G>C	c.291+1G>C	Splicing		3	N.A	Reported	34	N.A
FVII deficiency	F7	c.2T>C	p.Met1Thr	Start loss	1		Signal peptide	Reported	19.56	0.84719
FVII deficiency	F7	c.348del	p.Asn117Metfs*33	Frameshift	5		EGF1	New	27	N.A
FVII deficiency	F7	c.388del	p.Cys130Alafs*20	Frameshift	5		EGF1	Reported	33	N.A
FVII deficiency	F7	c.430+1G>A	c.430+1G>A	Splicing		5	N.A	Reported	33	N.A
FVII deficiency	F7	c.469G>T	p.Gly157Cys	Missense	6		EGF2	Reported	27.3	0.94428
FVII deficiency	F7	c.479A>G	p.Gln160Arg	Missense	6		EGF2	Reported	26	0.94653
FVII deficiency	F7	c.484T>C	p.Cys162Arg	Missense	6		EGF2	New	27.5	0.97852
FVII deficiency	F7	c.56T>C	p.Leu19Pro	Missense	1		Signal peptide	Reported	23.6	0.83526
FVII deficiency	F7	c.583T>C	p.Cys195Arg	Missense	7		Catalytic	Reported	28.6	0.98672
FVII deficiency	F7	c.635G>A	p.Arg212Gln	Missense	7		Catalytic	Reported	32	0.90721
FVII deficiency	F7	c.64+1G>C	c.64+1G>C	Splicing		1	N.A	New	33	N.A
FVII deficiency	F7	c.713G>A	p.Cys238Tyr	Missense	8		Catalytic	Reported	25.3	0.95175
FVII deficiency	F7	c.725T>C	p.Leu242Pro	Missense	8		Catalytic	New	25.1	0.90982

FVII deficiency	F7	c.752C>T	p.Ala251Val	Missense	8		Catalytic	Reported	23.8	0.95323
FVII deficiency	F7	c.783_799del	p.Trp261Cysfs*14	Frameshift	8		Catalytic	Reported	24.6	N.A
FVII deficiency	F7	c.790del	p.Leu264*	Frameshift	8		Catalytic	Reported	12.63	N.A
FVII deficiency	F7	c.805+5G>A	c.805+5G>A	Splicing		8	N.A	Reported	25.2	N.A
FVII deficiency	F7	c.817_831del	p.Leu273_Asp277del	Inframe Deletion	9		Catalytic	Reported	18.37	N.A
FVII deficiency	F7	c.847C>T	p.Arg283Trp	Missense	9		Catalytic	Reported	22.6	0.79704
FVII deficiency	F7	c.911C>T	p.Ala304Val	Missense	9		Catalytic	Reported	22.5	0.89099
FVII deficiency	F7	c.919C>T	p.Arg307Cys	Missense	9		Catalytic	Reported	24.2	0.83802
FVII deficiency	F7	c.934G>A	p.Val312Met	Missense	9		Catalytic	Reported	23.6	0.85249
FVII deficiency	F7	c.961C>T	p.Leu321Phe	Missense	9		Catalytic	Reported	23.5	0.84986
FVII deficiency	F7	c.970C>T	p.Pro324Ser	Missense	9		Catalytic	Reported	24.2	0.94952
FX deficiency	F10	c.1048G>A	p.Glu350Lys	Missense	8		Catalytic	Reported	26.5	0.88013
FX deficiency	F10	c.1087G>A	p.Gly363Ser	Missense	8		Catalytic	Reported	27.4	0.98816
FX deficiency	F10	c.1147C>T	p.Pro383Ser	Missense	8		Catalytic	Reported	25.5	0.94085
FX deficiency	F10	c.1169G>T	p.Cys390Phe	Missense	8		Catalytic	Reported	29.9	0.98955
FX deficiency	F10	c.119G>C	p.Arg40Thr	Missense	2		Signal peptide	Reported	24.4	0.94503
FX deficiency	F10	c.1216G>A	p.Gly406Ser	Missense	8		Catalytic	Reported	27.4	0.9706
FX deficiency	F10	c.1247A>T	p.Gln416Leu	Missense	8		Catalytic	Reported	29.1	0.95026
FX deficiency	F10	c.1262G>A	p.Gly421Asp	Missense	8		Catalytic	Reported	29.4	0.97512
FX deficiency	F10	c.128C>G	p.Ser43Cys	Missense	2		Gla	Reported	22.2	0.87771
FX deficiency	F10	c.139delG	p.Glu47Argfs*2	Frameshift	2		Gla	Reported	27.2	N.A
FX deficiency	F10	c.1465T>C	p.*489Argext*?	Stop lost	8		Catalytic	New	7.54	N.A
FX deficiency	F10	c.161_162del	p.Glu56Valfs*16	Frameshift	2		Gla	New	24.6	N.A
FX deficiency	F10	c.202C>T	p.Arg68Cys	Missense	2		Gla	Reported	24.3	0.89238
FX deficiency	F10	c.205G>A	p.Glu69Lys	Missense	2		Gla	Reported	26.1	0.99157
FX deficiency	F10	c.232-3T>G	c.232-3T>G	Splicing		2	N.A	Reported	23.5	N.A
FX deficiency	F10	c.307G>C	p.Asp103His	Missense	4		EGF	Reported	26.8	0.97662
FX deficiency	F10	c.353G>A	p.Gly118Asp	Missense	4		EGF	Reported	26.9	0.99982
FX deficiency	F10	c.362G>A	p.Cys121Tyr	Missense	4		EGF	Reported	27.2	0.99647
FX deficiency	F10	c.370+2T>C	c.370+2T>C	Splicing		4	N.A	New	33	N.A
FX deficiency	F10	c.400G>A	p.Gly134Arg	Missense	5		EGF	Reported	30	0.98708
FX deficiency	F10	c.[400G>A;405C>A]	p.Gly134Arg_p.Asp135Glu	Missense_Missense	5		EGF	Reported		N.A
FX deficiency	F10	c.513dup	p.Cys172Leufs*25	Frameshift	6		Catalytic	New	26.7	0
FX deficiency	F10	c.517G>T	p.Gly173Trp	Missense	6		Catalytic	Reported	25.6	0.94466
FX deficiency	F10	c.526_558del	p.Thr176_Gln186del	Inframe Deletion	6		Catalytic	Reported	17.05	N.A
FX deficiency	F10	c.61G>A	p.Gly21Arg	Missense	1		Signal peptide	Reported	22.4	0.79704
FX deficiency	F10	c.70+3A>G	c.70+3A>G	Splicing		1	N.A	Reported	9.747	N.A
FX deficiency	F10	c.730G>A	p.Gly244Arg	Missense	7		Catalytic	Reported	26.9	0.96354
FX deficiency	F10	c.785G>A	p.Gly262Asp	Missense	7		Catalytic	Reported	24.9	0.99718
FX deficiency	F10	c.910G>A	p.Glu304Lys	Missense	8		Catalytic	Reported	17.5	0.79704
FX deficiency	F10	c.969C>G	p.Ile323Met	Missense	8		Catalytic	Reported	22.7	0.91112
FX deficiency	F10	Chr_deletion	Chr_deletion	large deletion			N.A	New		N.A
FXI deficiency	F11	c.1079T>C	p.Leu360Pro	Missense	10		Apple4	Reported	23.4	0.89422
FXI deficiency	F11	c.1112T>A	p.Leu371*	Stop gained	10		Apple4	Reported	36	N.A
FXI deficiency	F11	c.1169G>C	p.Gly390Ala	Missense	11		Catalytic	Reported	24.3	0
FXI deficiency	F11	c.1693G>A	p.Glu565Lys	Missense	14		Catalytic	Reported	23.2	0.86886
FXI deficiency	F11	c.16C>T	p.Gln6*	Stop gained	2		Signal peptide	Reported	35	N.A
FXI deficiency	F11	c.1778C>T	p.Thr593Met	Missense	15		Catalytic	Reported	25.1	0.9721
FXI deficiency	F11	c.1837G>A	p.Glu613Lys	Missense	15		Catalytic	Reported	14.67	0.66297
FXI deficiency	F11	c.1840T>G	p.Tyr614Asp	Missense	15		Catalytic	Reported	25.2	0.94803
FXI deficiency	F11	c.1856T>C	p.Leu619Pro	Missense	15		Catalytic	New	25.8	0.92087

FXI deficiency	F11	c.325+1G>A	c.325+1G>A	Splicing		4	N.A	Reported	33	
FXI deficiency	F11	c.403G>T	p.Glu135*	Stop gained	5		Apple2	Reported	36	N.A
FXI deficiency	F11	c.408C>A	p.Cys136*	Stop gained	5		Apple2	Reported	35	N.A
FXI deficiency	F11	c.486-14T>C	c.486-14T>C	Splicing		5	N.A	New	8.166	N.A
FXI deficiency	F11	c.495T>A	p.Cys147*	Stop gained	6		Apple2	New	35	N.A
FXI deficiency	F11	c.595+3A>G	c.595+3A>G	Splicing		6	N.A	Reported	22.6	N.A
FXI deficiency	F11	c.815_820del	p.Ser272_Ala274delinsThr	Inframe Deletion	8		Apple3	Reported	11.74	N.A
FXI deficiency	F11	c.901T>C	p.Phe301Leu	Missense	9		Apple4	Reported	25.9	0.94162
FXI deficiency	F11	c.943G>A	p.Glu297Lys	Missense	9		Apple4	Reported	17.41	0.81888
FXI deficiency	F11	c.961_962del	p.Cys321Hisfs*37	Frameshift	9		Apple4	Reported	26.5	N.A
FXIII deficiency	F13A	c.1047G>T	p.Leu349Phe	Missense	8		Catalytic	New	24.8	0
FXIII deficiency	F13A	c.1142T>A	p.Met381Lys	Stop gained	9		Catalytic	Reported	28.1	0.99803
FXIII deficiency	F13A	c.1149G>T	p.Arg382Ser	Missense	9		Catalytic	Reported	23.3	0.95433
FXIII deficiency	F13A	c.1212C>G	p.Ser404Arg	Missense	9		Catalytic	New	18.9	0.84019
FXIII deficiency	F13A	c.1226G>A	p.Arg409Gln	Missense	10		Catalytic	Reported	27.9	0.75004
FXIII deficiency	F13A	c.1259_1262dupACGG	p.His422Argfs*8	Frameshift	10		Catalytic	New	32	N.A
FXIII deficiency	F13A	c.1459+1G>A	c.1459+1G>A	Splicing		11	N.A	Reported	33	N.A
FXIII deficiency	F13A	c.1475_1476del	p.Arg492Ilefs*12	Frameshift	12		Catalytic	Reported	32	N.A
FXIII deficiency	F13A	c.1730C>T	p.Thr577Met	Missense	12		βbarrel1	New	0.864	0.70684
FXIII deficiency	F13A	c.1741del	p.Leu581Cysfs*9	Frameshift	12		βbarrel1	New	24.3	N.A
FXIII deficiency	F13A	c.1984C>T	p.Arg661*	Stop gained	14		βbarrel2	Reported	36	N.A
FXIII deficiency	F13A	c.2046-1G>A	c.2046-1G>A	Splicing		14	N.A	Reported	33	N.A
FXIII deficiency	F13A	c.307G>A	p.Glu103Lys	Stop gained	3		βsandwich	Reported	28.1	0.93543
FXIII deficiency	F13A	c.320G>A	p.Arg77His	Missense	4		βsandwich	Reported	29.6	0.98003
FXIII deficiency	F13A	c.562T>C	p.Trp188Arg	Missense	4		Catalytic	Reported	31	0.97662
FXIII deficiency	F13A	c.602del	p.Arg202Glufs*6	Frameshift	5		Catalytic	Reported	29.4	N.A
FXIII deficiency	F13A	c.646G>A	p.Gly216Arg	Missense	5		Catalytic	Reported	28.9	0.99405
FXIII deficiency	F13A	c.691-1G>A	c.691-1G>A	Splicing		5	N.A	Reported	35	N.A
FXIII deficiency	F13A	c.787G>A	p.Gly263Arg	Missense	6		Catalytic	Reported	25.9	0.82799
FXIII deficiency	F13A	c.791C>T	p.Ser263Phe	Missense	6		Catalytic	Reported	32	0.99058
FXIII deficiency	F13A	c.868C>T	p.Arg260Cys	Missense	7		Catalytic	Reported	26.6	0.99124

- N.A, Not available

- A CADD score >20 is considered pathogenic

- The REVEL score for a missense variant ranges from 0 to 1, with scores ≥ 0.5 considered more likely to be pathogenic

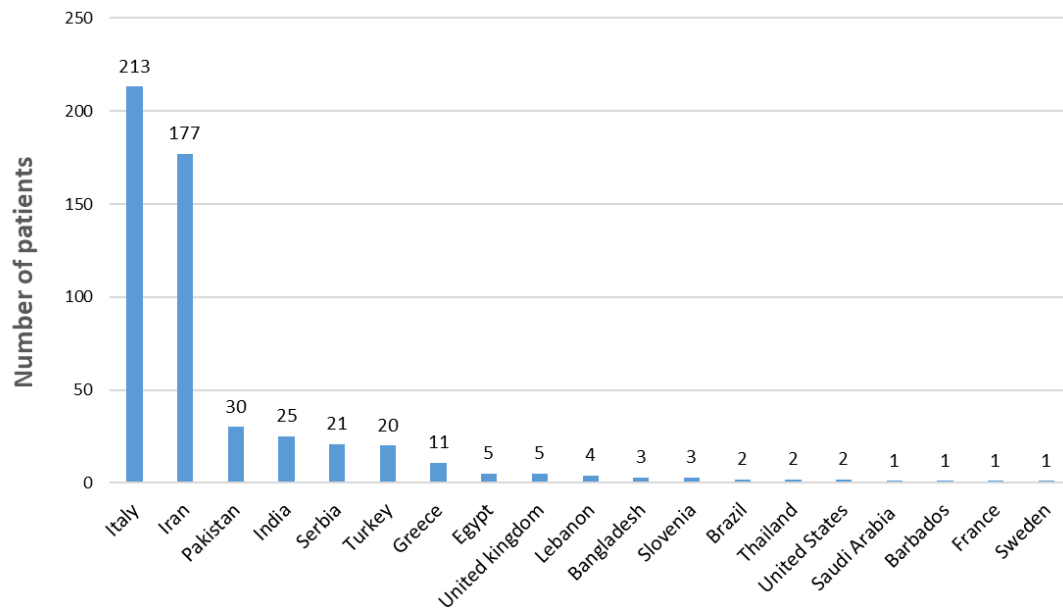
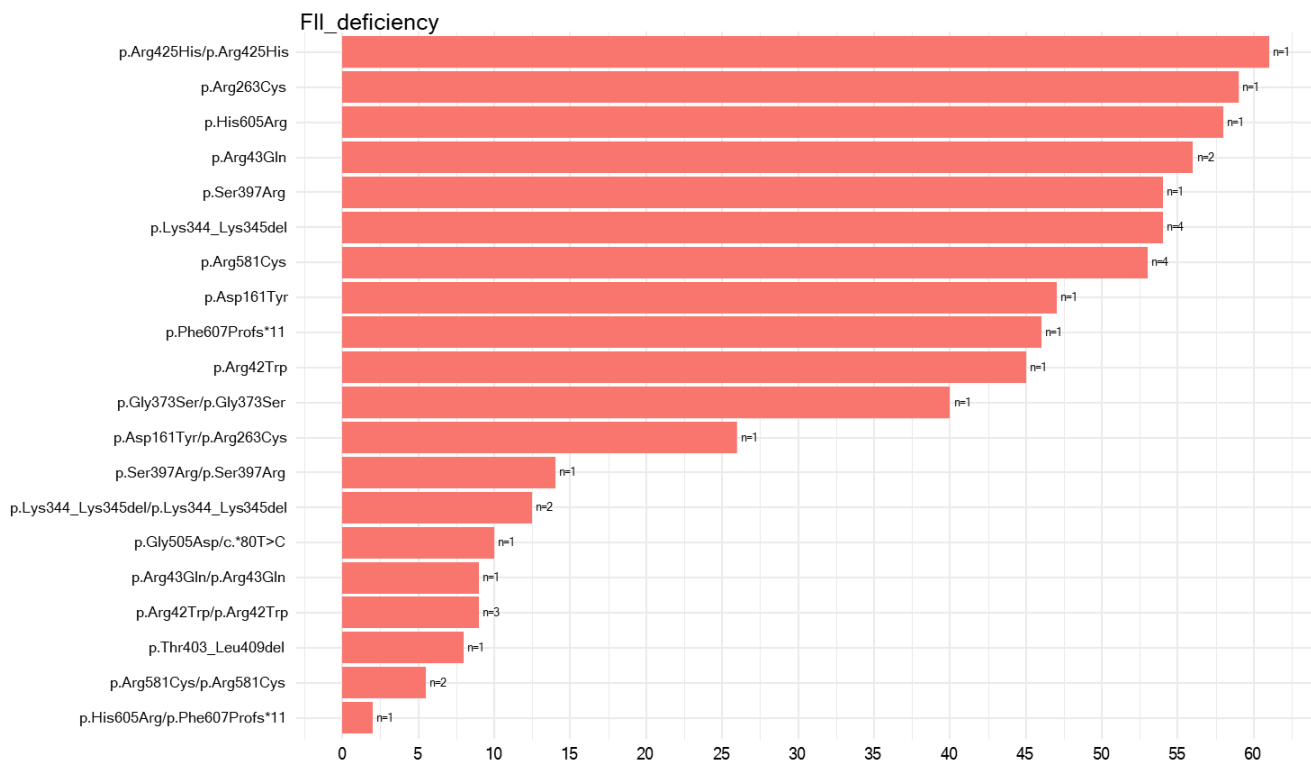
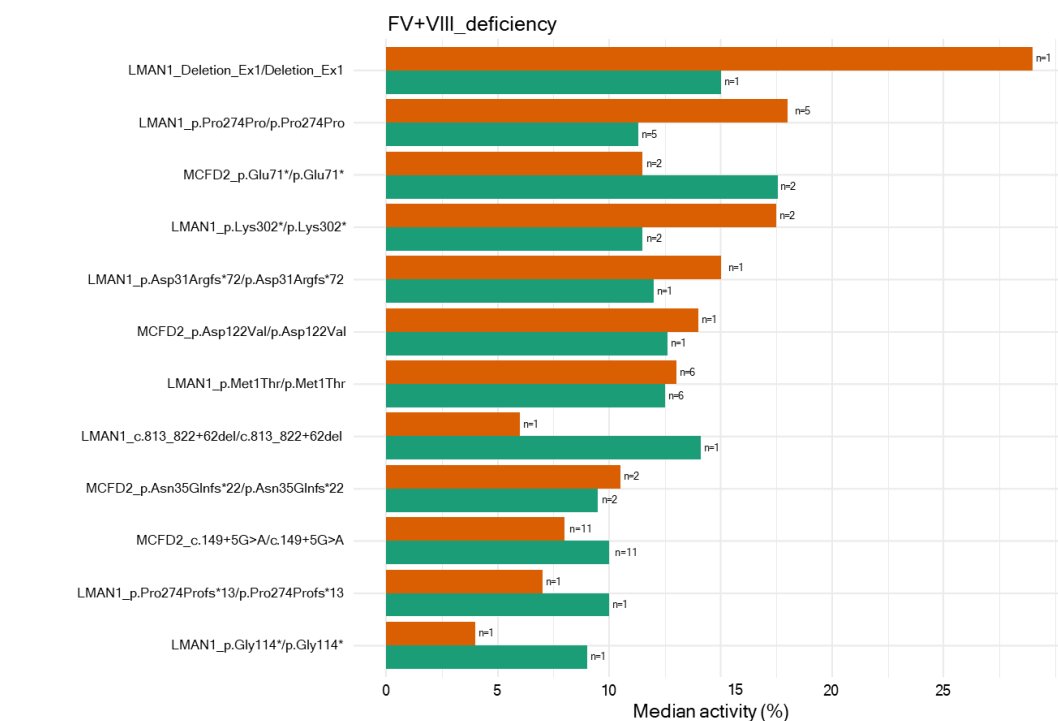
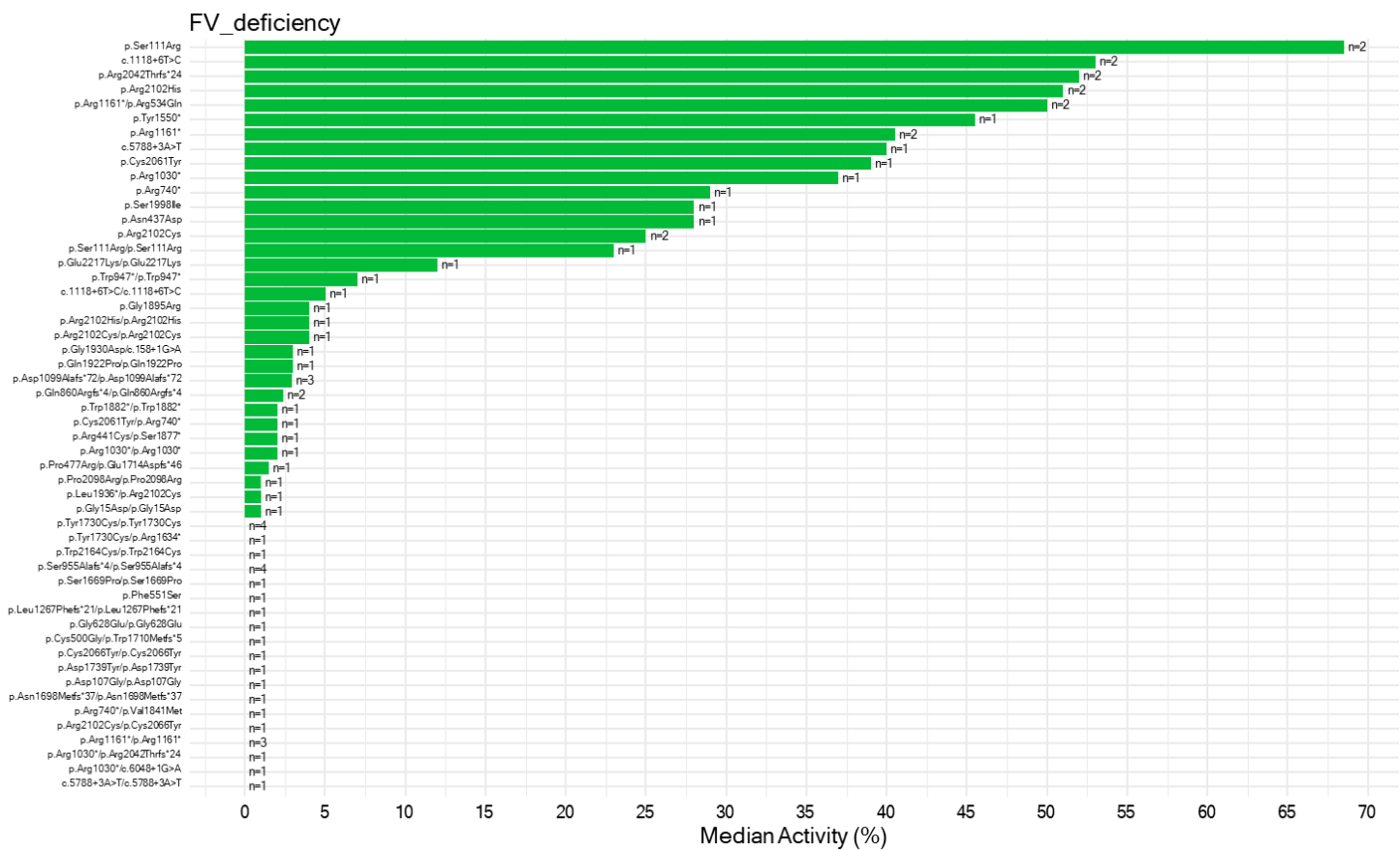
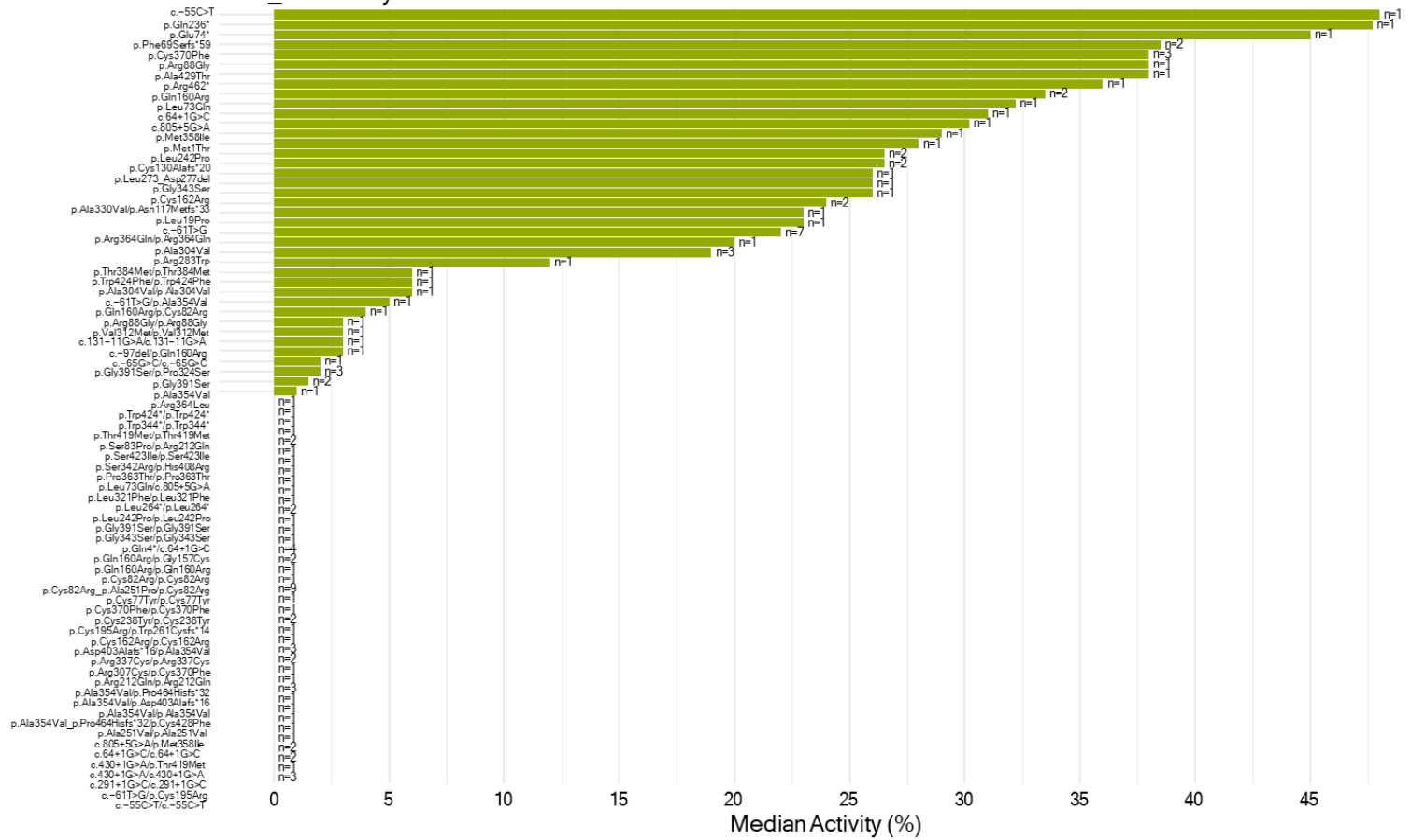


Figure S1. Country distribution of study population.

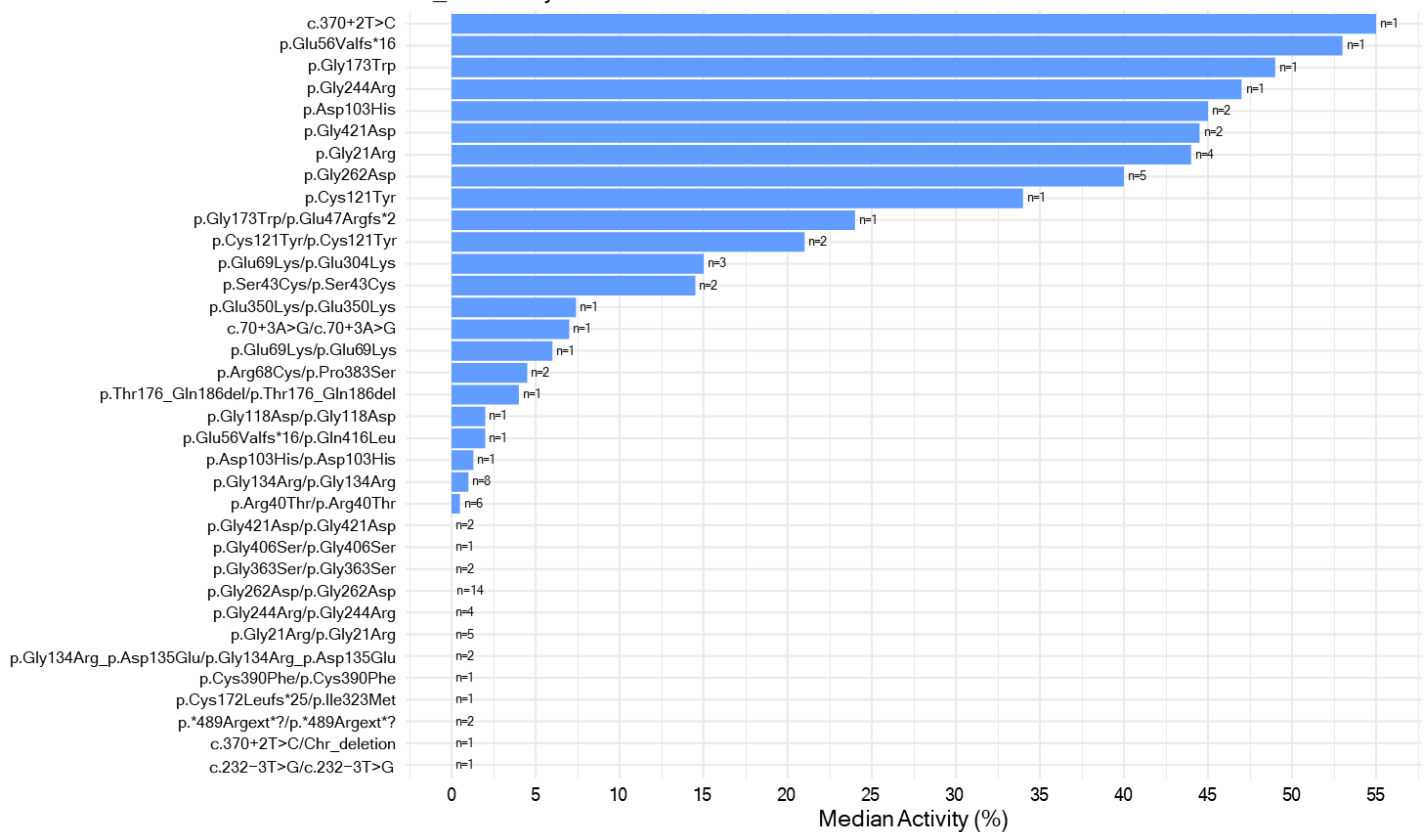




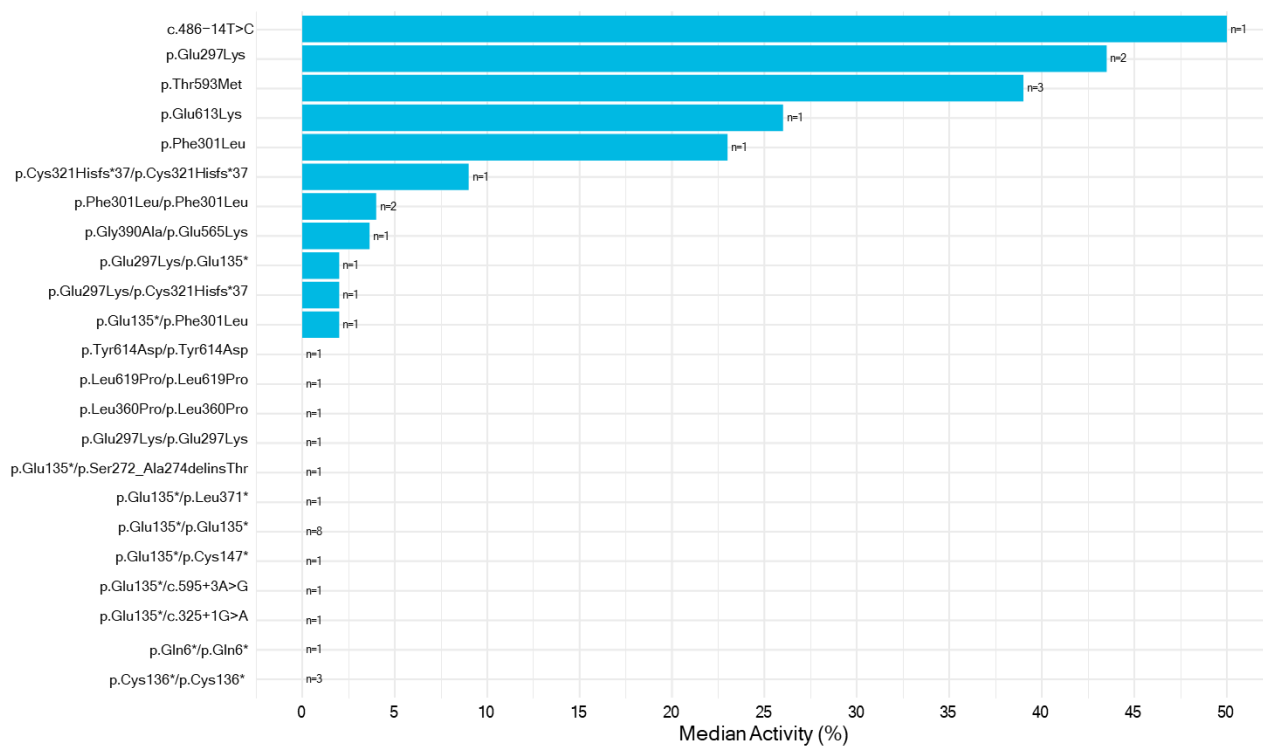
FVII_deficiency



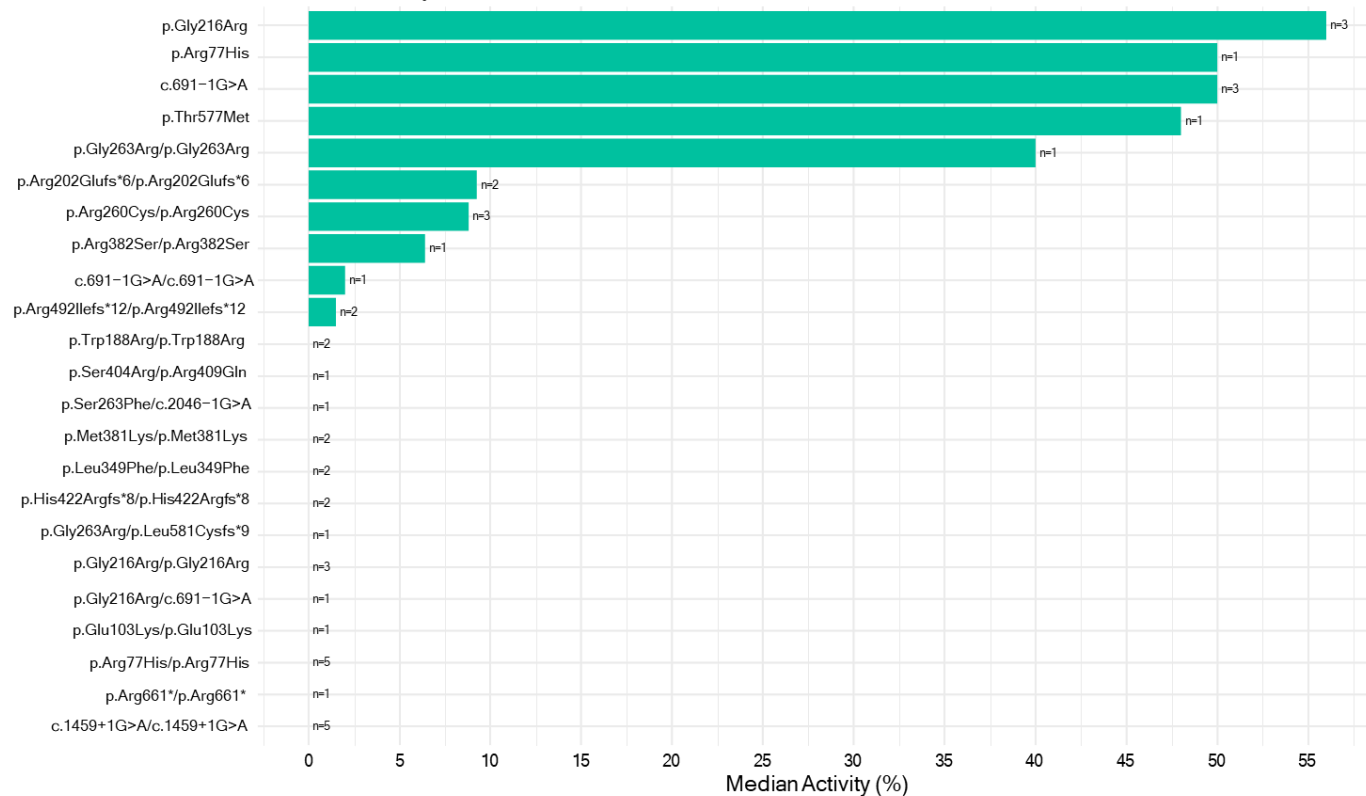
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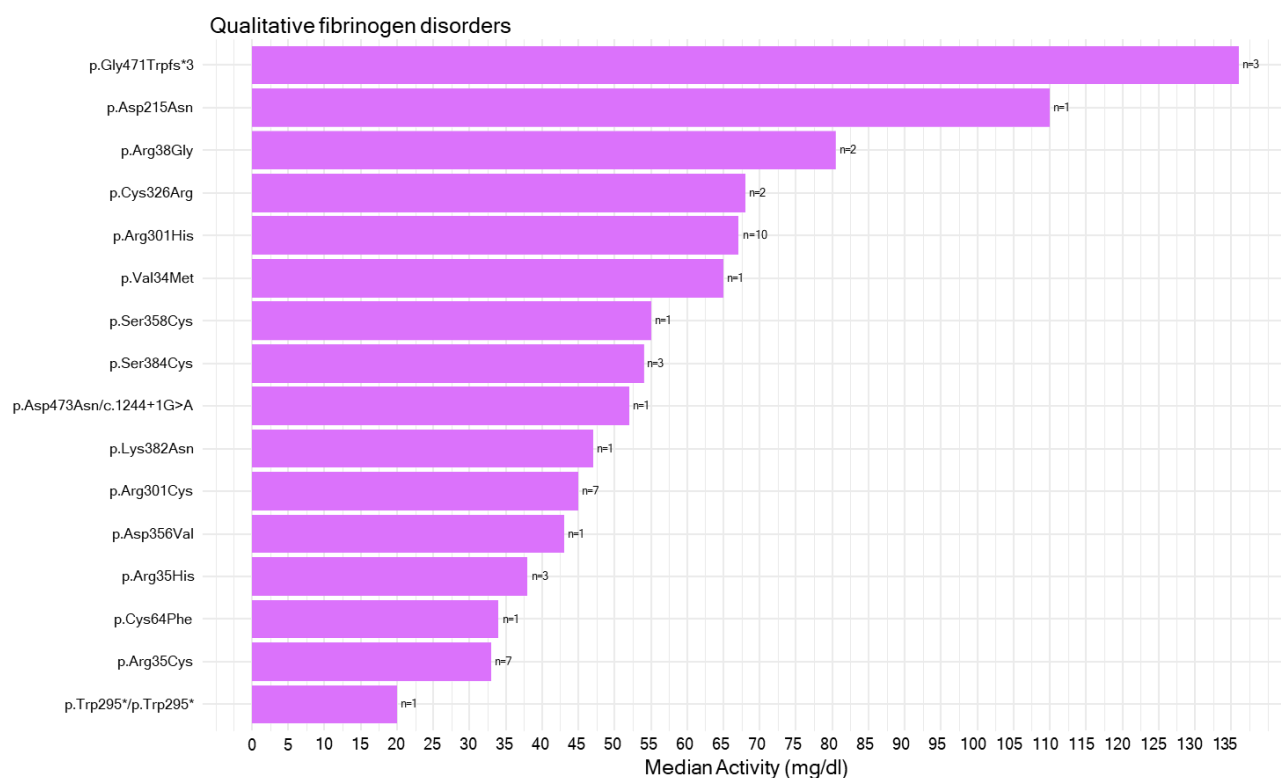


FXI_deficiency



FXIII_deficiency





Quantitative fibrinogen disorders

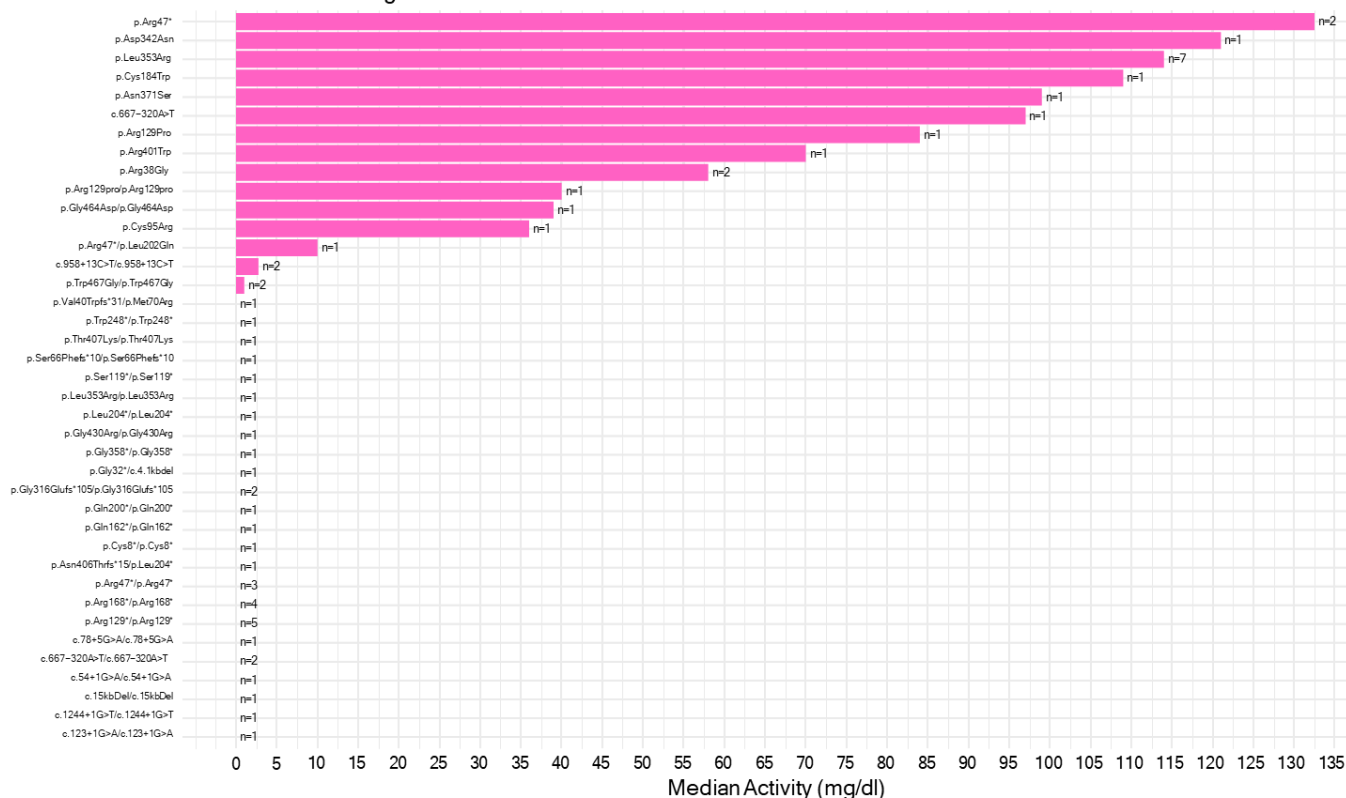


Figure S2. Coagulation factor activity levels for individual identified variants. The bar chart illustrates the median factor activity levels for each variant in affected cases. The normal factor activity levels are as follows: Fibrinogen: 150-450 mg/dl, FII: 70-120%, FV: 70-140%, FVII: 62-140%, FX: 66-130%, FXI: 70-140%, FXIII: 59-181%.

Chapter II

Diagnostic utility of bleeding assessment tools in congenital fibrinogen deficiencies

Samin Mohsenian¹, Omid Seidizadeh¹, Roberta Palla¹, Mohammad Jazebi², Azita Azarkeivan³, Samira Moazezi², Mohammad Reza Baghaipour², Marzia Menegatti⁴, Flora Peyvandi^{1, 4}

1. Università degli Studi di Milano, Department of Pathophysiology and Transplantation, Milan, Italy
2. Iranian Comprehensive Hemophilia Care Center, Tehran, Iran
3. Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran
4. Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center and Fondazione Luigi Villa, Milan, Italy

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Abstract

Background: The assessment of clinical history is crucial before referring a patient for further laboratory testing. Bleeding assessment tools (BAT) are developed to standardize clinical evaluation. A small number of patients with congenital fibrinogen deficiencies (CFDs) have been evaluated with these tools without definitive results.

Aims: We compared the adequacy of the ISTH-BAT and the European network of rare bleeding disorders bleeding score system (EN-RBD-BSS) to identify patients with CFDs. The correlation between the two BATs and fibrinogen levels and patient clinical grade severity was further analyzed.

Methods: We included 100 Iranian patients with CFDs. Routine coagulation and fibrinogen-specific tests (fibrinogen antigen [Fg:Ag] and activity [Fg:C]) were performed. The ISTH-BAT and EN-RBD-BSS were used to assess the bleeding score (BS) of all patients.

Results: The ISTH-BAT and EN-RBD-BSS median (range) were 4 (0-16) and 2.21 (-1.49-6.71), with a statistically significant moderate correlation between the two systems ($r=0.597$, $P<0.001$). In patients with quantitative deficiencies (afibrinogenemia and hypofibrinogenemia), the correlation between Fg:C and the ISTH-BAT was moderately negative ($r= -0.4$, $P<0.001$), while the correlation between Fg:C and the EN-RBD-BSS was weakly negative ($r= -0.38$, $P<0.001$). Overall, 70% and 72% of patients with fibrinogen deficiencies were correctly identified by both the ISTH-BAT and EN-RBD-BSS, respectively.

Conclusion: These results suggest that in addition to the ISTH-BAT, the EN-RBD-BSS may also be useful in identifying CFD patients. We found a significant level of sensitivity for detecting fibrinogen deficiency in the two BATs, and bleeding severity classification correctly identified severity grades in almost two-thirds of patients.

Introduction

Congenital fibrinogen disorders (CFDs) that account for almost 8% of rare bleeding disorders (RBDs), are classified based on the fibrinogen plasma activity (Fg:C) and antigen levels (Fg:Ag). CFDs are classified into quantitative (afibrinogenemia and hypofibrinogenemia) or qualitative (dysfibrinogenemia and hypodysfibrinogenemia) deficiencies [1, 2] and present a wide spectrum of clinical manifestations ranging from asymptomatic to major bleeding with even thrombosis events [3, 4]. Patients with congenital afibrinogenemia show very low or undetectable fibrinogen levels causing severe bleeding symptoms and venous or arterial thrombosis, although to a lesser extent than bleeding [5, 6]. Inherited hypofibrinogenemia, due to a proportional decrease of the functional and antigenic levels of fibrinogen, is associated with a mild bleeding tendency and more rarely thrombotic complications [7, 8]. Inherited dysfibrinogenemia is characterized by low Fg:C but normal Fg:Ag plasma levels [3]. Most dysfibrinogenemic patients are reported to be symptom-free, others present with bleeding and thrombosis manifestations [9, 10]. Hypodysfibrinogenemia is defined by disproportionately decreased plasma levels of Fg:C and Fg:Ag and clinical features can be more severe than in dysfibrinogenemia with a risk of thrombosis [7].

The evaluation of clinical history is mandatory to direct a patient suspected of having bleeding disorders toward further laboratory investigation. Therefore since 1982 bleeding assessment tools (BAT) were developed to address this need and standardize the assessment of bleeding symptoms [11]. A standard bleeding questionnaire was first developed in 2005 to assess the clinical presentation of patients with von Willebrand disease (VWD). In 2010, the joint VWF and pediatric hemostasis Scientific and Standardization Committees (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) developed and published the revised BAT system (ISTH-BAT) to address the description of bleeding symptoms or diagnosis of bleeding disorders [12, 13]. Based on the available data, the ISTH-BAT normal range is 0–3 for adult males, 0–5 for adult females and 0–2 for children. Therefore, the cut-off for a positive or abnormal BS is ≥ 4 in adult males, ≥ 6 in adult females and ≥ 3 in children, and these thresholds should help to distinguish patients with bleeding disorders than normal subjects [14].

However, only a limited number of patients with RBDs were evaluated using these tools, and no conclusive results have been obtained [15–17]. In 2012, Peyvandi et al conducted a study on behalf of the European Network of Rare Bleeding Disorders (EN-RBD) in order to examine the relationship between coagulation factor activity level and bleeding severity in patients with RBDs. Accordingly,

patients with RBDs were classified into four categories based on their bleeding location, potential clinical impact and spontaneous bleeding (asymptomatic, grade I, grade II and grade III) [18].

More recently and within the same project, Palla et al developed a novel bleeding score system (BSS) dedicated to RBDs (EN-RBD-BSS, from now on), which enlists previous bleeding symptoms, frequency, spontaneity, extent, localization and relationship with prophylaxis and acute treatment [19]. In addition, the type of surgery is considered including bleeding during surgery (minor and major) and tonsillectomy. Although the ISTH-BAT cut-off values differ based on gender and age [14], the cut-off value for an abnormal EN-RBD-BSS is ≥ 1.5 for men, women and children. Unlike the previous BATs, sex and age are among the variables that have been included in the total bleeding score calculation of the EN-RBD-BSS. This new BSS has been shown to be useful in identifying patients who have to be followed for further laboratory investigations [19].

Although the utility of such BS tools has been examined in cohorts including all RBDs [17, 19], no studies have been conducted specifically on CFD because of its low prevalence, which prevents large cohorts from being available. Thus, we aimed to evaluate the diagnostic utility of the ISTH-BAT and the EN-RBD-BSS in a large well-characterized cohort of patients with CFDs. In addition, the correlation between the two BAT systems with plasma fibrinogen levels and clinical severity classification was analyzed.

Materials and methods

Patients

A total of 164 Iranian patients with a pre-existing diagnosis of CFDs were included in this study. The diagnosis of patients was based on low Fg:C and Fg:Ag plasma levels. The study population was selected from the referred patients to the Iranian Comprehensive Hemophilia Care Center (ICHCC), Tehran. This study was approved by the ethics Committee of the ICHCC and the High Institute for Research and Education in Transfusion Medicine. Informed written consent was obtained from all participating patients according to the Helsinki Declaration.

Bleeding scores

The ISTH-BAT and EN-RBD-BSS were used to quantify the bleeding manifestations. The ISTH-BAT includes 14 questions regarding clinical symptoms and each bleeding symptom can be scored ranging from 0 to 4 [13]. To complete these questionnaires, newly diagnosed participants were asked about the type, severity, frequency, and need for treatment of their clinical symptoms. We also calculated the bleeding scores according to the recorded clinical data at the age of diagnosis of

previously diagnosed cases. The EN-RBD-BSS questionnaire comprises 15 parameters, including sex, age, 13 clinical symptoms and sub-questions such as severity and spontaneity of bleeding as well as treatment and prophylaxis [19].

Laboratory assays

All samples were collected in 0.105M buffered trisodium citrate solution and centrifuged at 2700× g for 15 minutes at room temperature. The plasma sample was then aliquoted and stored at -80° C for further assays. The fresh samples were used for routine coagulation tests and fresh or frozen plasma samples were used to measure fibrinogen levels. Routine coagulation screening assays including activated partial thromboplastin time [APTT], prothrombin time [PT], thrombin time [TT] and Fg:C assay (Clauss method, Technoclone GmbH, Austria Brunner Str. 67 - 1230 Vienna) were performed by the Sysmex CA-1500 fully automated system. Fg:Ag level was measured based on the turbidimetric latex immunoassay method (HYPHEN BioMed, LIAPHEN, Neuville-sur-Oise, France) by Sysmex CS-2400 fully automated system.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 26. Values are reported as medians (range). Pearson's correlation was performed to evaluate correlations between: (1) the ISTH-BAT and EN-RBD-BSS, (2) the two score system values and the baseline Fg:C as well as Fg:Ag levels (3), and Fg:C and clinical bleeding severity. The correlation strength was classified as follows: very weak correlation ($\pm 0.2 >$), weak correlation (± 0.2 to $\pm 0.4 >$), moderate correlation (± 0.4 to $\pm 0.6 >$), strong correlation (± 0.6 to $\pm 0.8 >$), or very strong correlation (± 0.8 to ± 1) [20]. Furthermore, a sensitivity analysis was performed using half of the lowest detection limit (i.e. 5 mg/dl for a detection limit of 10 mg/dl) for afibrinogenemic cases. For comparing medians between two independent groups and four groups on a dependent variable, the Mann-Whitney U test and the Kruskal–Wallis test were used, with a *P-value* <0.05 considered statistically significant.

Results

Patients

Out of 164 patients with a pre-existing diagnosis of CFD [21], a total number of 100 patients had complete laboratory and clinical data and could be included in this study. The mean ($\pm$ SD) age of the study population at the time of diagnosis (n=100) was 18.5 ($\pm$ 15.9) that 46 were adults and 54 children. The characteristics, clinical and laboratory results of patients are summarized in Table 1.

Table 1. Characteristics of the study cohort

Type	N (%)	Age of diagnosis (mean±SD)	Adult female, N (%)	Adult male, N (%)	Children female, N (%)	Children male, N (%)	PT (s)	PTT (s)	TT (s)	Clauss (mg/dl) **	Ag (mg/dl) **	ISTH-BAT	EN-RBD-BSS
Afibrinogenemia	58 (58%)	13.3 (±11.9)	11 (38%)	9	19	19	*>60	*>120	*>60	Un-detectable	Un-detectable	5 (0-16)	2.64 (-0.09- 6.71)
Hypo ^β	9 (9%)	18.5 (±14.1)	3 (10%)	2	2	2	14 (11-19)	38 (31-47)	24 (23-38)	90 (75-130)	105 (73-126)	3 (0-7)	1.41 (0.51- 4.11)
Dys ^β	24 (24%)	28.2 (±18.8)	11 (38%)	4	4	5	16 (12-21)	37 (32-47)	34 (19-46)	48 (0-78)	292 (183-370)	3 (0-6)	1.31 (-1.19- 2.53)
Hypodys ^β	9 (9%)	25.4 (±19.3)	4 (14%)	2	1	2	15 (11-20)	37 (30-180)	25 (17-39)	42 (7-90)	111 (76-156)	5 (2-8)	2.21 (-1.49- 4.22)
All patients	100	18.5 (±15.9)	29 (29%)	17	26	28	-	-	-	41 (0-130)	126 (0-370)	4 (0-16)	2.21 (-1.49- 6.71)

The laboratory and bleeding score results are reported as median (range). PT, Prothrombin time; APTT, Activated partial thromboplastin time; ISTH-BAT, International Society on Thrombosis and Haemostasis-Bleeding Assessment Tool; EN-RBD-BSS, European network of rare bleeding disorders-bleeding score system. *Infinitely prolonged. **The normal range of Clauss and antigen is 150-450 mg/dl. ^βHypo, hypofibrinogenemia; Dys, dysfibrinogenemia; Hypodys, hypodysfibrinogenemia.

Clinical presentation

Among the entire study population, 94% of cases experienced at least one bleeding episode during their life. A thrombosis event was observed in a hypodysfibrinogenemic case among our study population. As shown in Figure 1, the most common bleeding manifestation among all patients was cutaneous bleeding, being reported in 50% of cases, while hematuria was observed only in one patient (1%). Further analysis of bleeding presentations specific to each fibrinogen deficiency subgroup was performed. Umbilical cord bleeding was the most common bleeding symptom in afibrinogenemic cases and cutaneous bleeding was mostly seen in hypofibrinogenemic and hypodysfibrinogenemic cases. Most patients with dysfibrinogenemia experienced menorrhagia and cutaneous bleeding, in fact, menorrhagia was mostly observed in qualitative fibrinogen deficiencies (Supplementary Figure 1). Among females of fertile age, 12 of 36 (33%) cases had menorrhagia. There were 14 pregnancies, of which 11 were delivered and 3 (21%) ended in spontaneous abortions. Among 11 deliveries, one

was uneventful, two were premature but with successful childbirth, and the remaining 8 were in the term. We found postpartum hemorrhage in 3 patients (27%).

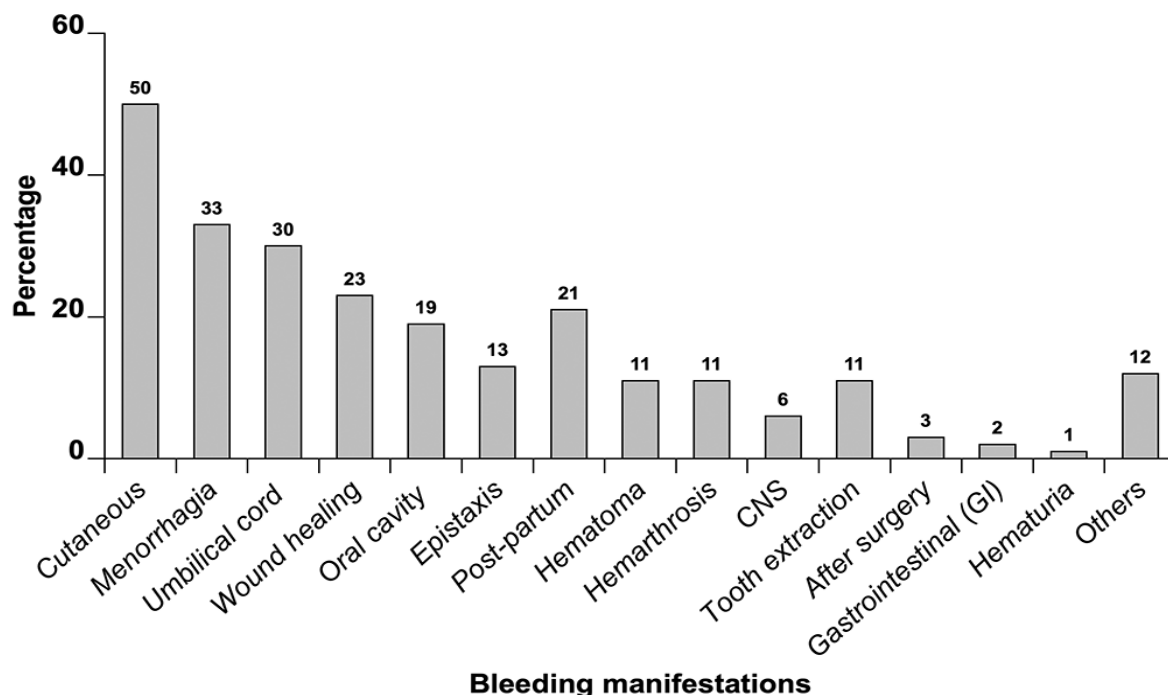


Figure 1. Reported frequency of different types of bleeding symptoms. Menorrhagia was considered in women who were in fertile age, and postpartum bleeding was assessed in women who underwent labor. Others bleeding: circumcision (9 afibrinogenemic cases), ruptured ovarian cyst (2 afibrinogenemic cases), hyphema (1 dysfibrinogenemic case); CNS, Central Nervous System.

Laboratory assays

The normal ranges of laboratory tests were established in a cohort of 50 healthy subjects and were determined to be 10-13 s for PT, 18-28 s for APTT and 14-19 s for TT. The normal ranges of Fg:C and Fg:Ag were 154-475 mg/dl and 184-334 mg/dl, respectively. Patients with afibrinogenemia had infinitely prolonged conventional testing and showed undetectable levels of Fg:C and Fg:Ag. Patients with dysfibrinogenemia had the most prolonged PT and TT, low Fg:C level and normal Fg:Ag level. Patients with hypodysfibrinogenemia had reduced fibrinogen levels with a disproportionate Fg:C /Fg:Ag ratio (Table 1).

Bleeding scores

ISTH-BAT

The overall median (range) of the ISTH-BAT in the whole group was 4 (0-16). Males and females showed the same ISTH-BAT score (5, $P=0.58$), children had a lower median score than adults (4 vs.

5, $P = 0.06$). Patients with afibrinogenemia and hypodysfibrinogenemia had the highest median scores for the ISTH-BAT of 5 (0-16) and 5 (2-8), respectively (Figure 2). However, lower median scores were found for hypofibrinogenemia and dysfibrinogenemia (median 3, range 0-7 and median 3, range 0-6). The overall correlation between the ISTH-BAT score and Fg:C was moderately negative ($r = -0.42$, $P < 0.001$), comparable to that of Fg:Ag ($r = -0.407$, $P < 0.001$, Figure 3A). Regarding quantitative fibrinogen deficiencies (afibrinogenemia and hypofibrinogenemia), the correlation between Fg:C and the ISTH-BAT score was moderately negative ($r = -0.4$, $P < 0.001$, Supplementary Figure 2).

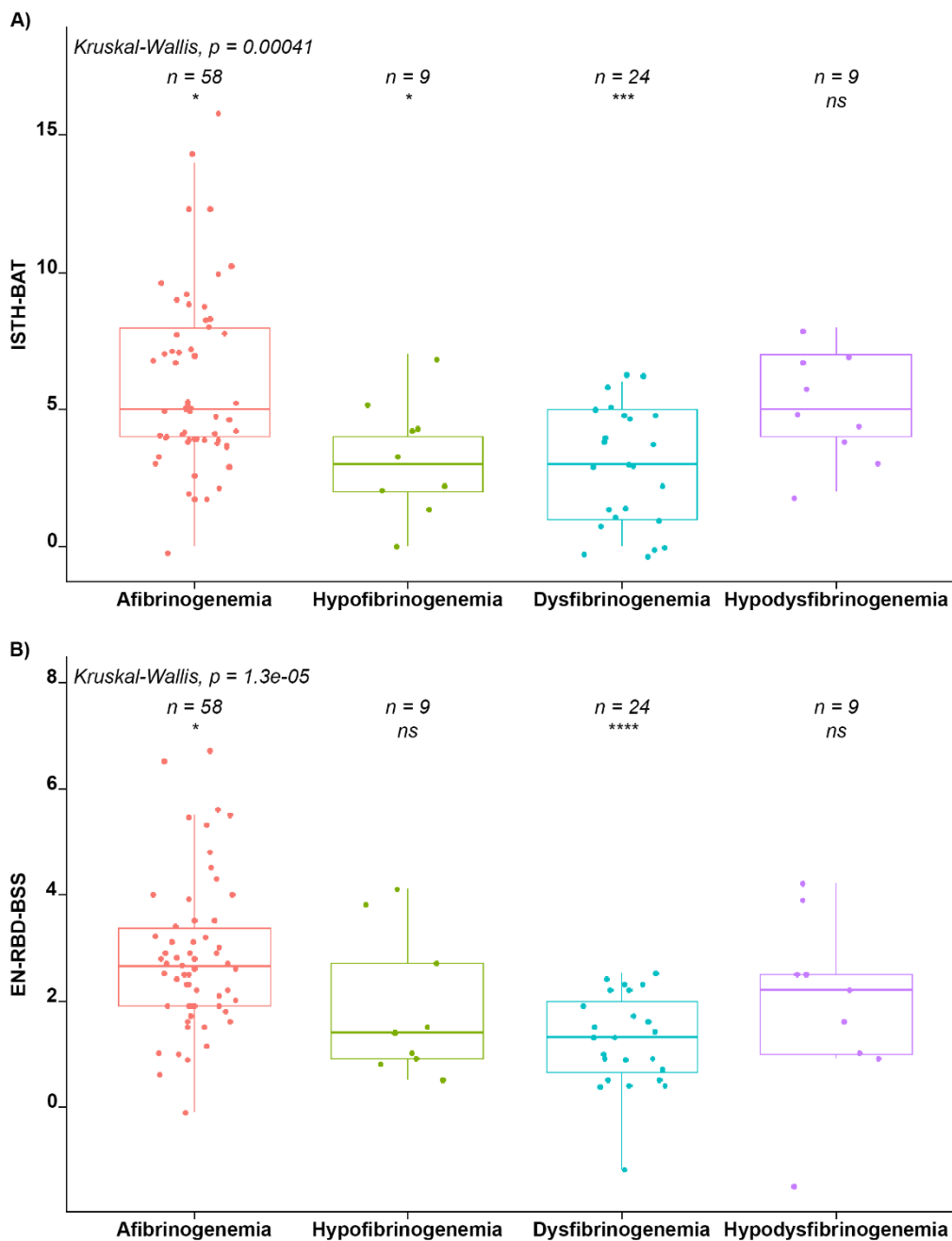


Figure 2. Box plot of the ISTH-BAT and EN-RBD-BSS scores in patients with fibrinogen deficiency. The box plot solid line indicates the median. The Kruskal-Wallis test was performed to test differences in the ISTH-BAT and EN-RBD-BSS between different subclasses of fibrinogen disorders. * ($p\text{-value} < 0.05$), ** ($p\text{-value} < 0.01$), *** ($p\text{-value} < 0.001$), and **** ($p\text{-value} < 0.0001$).

EN-RBD-BSS

The median (range) of the EN-RBD-BSS was 2.21 (-1.49- 6.71) with a median of 2.21 (-1.49- 5.50) for females, 2.05 (-0.09- 6.71) for males and 2.05 (0.51- 5.47) for children. The highest EN-RBD-BSS was found in patients with afibrinogenemia (2.64, range -0.09- 6.71) and in hypodysfibrinogenemia (2.21, range -1.49- 4.22), whereas dysfibrinogenemic patients had the lowest BSS (1.31, range -1.19- 2.53). Patients with hypofibrinogenemia had a BSS median of 1.41 (0.51- 4.11). The overall correlation between the EN-RBD-BSS score and Fg:C was moderately negative ($r = -0.446$, $P < 0.001$) and was comparable to that of Fg:Ag ($r = -0.466$, $P < 0.001$, Figure 3A). A weak but significant negative correlation was also found between Fg:C and EN-RBD-BSS score ($r = -0.38$, $P < 0.001$) in patients with quantitative fibrinogen deficiencies (Figure S2).

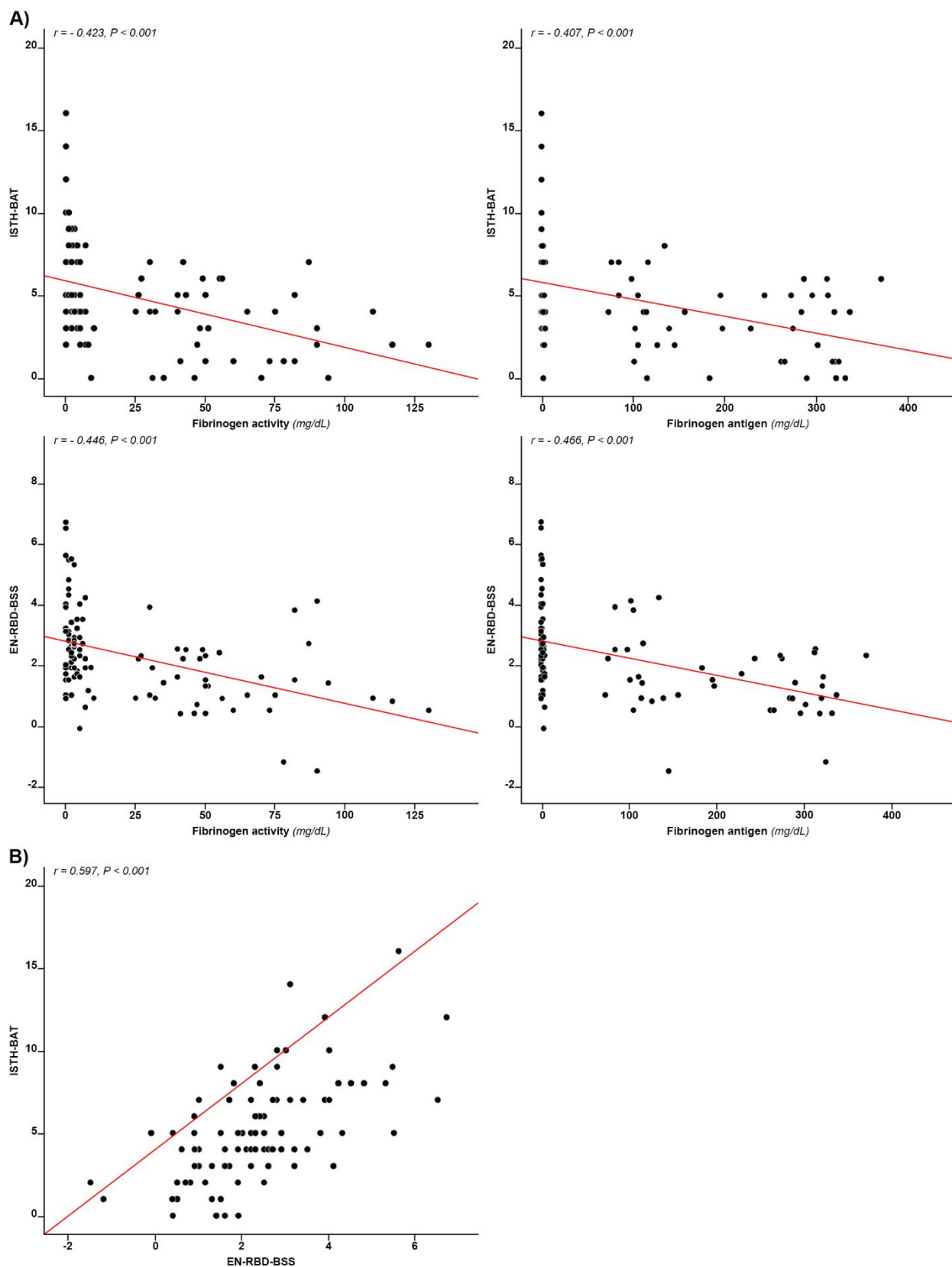


Figure 3. A. Correlation between fibrinogen activity and antigen levels, and bleeding assessment tools with linear regression lines. **B.** Correlation between the ISTH-BAT and EN-RBD-BSS in patients with fibrinogen deficiencies.

Correlation between ISTH-BAT and EN-RBD-BSS

We found a moderate positive correlation ($r = 0.597$, $P < 0.001$) between ISTH-BAT and EN-RBD-BSS (Figure 3B) with the strongest correlation found in hypodysfibrinogenemic patients ($r = 0.916$, $P = 0.001$). Taking as a reference, the established cut-off value for the abnormal bleeding score for the two scoring systems [14, 19], overall 70% and 72% of cases were identified as being fibrinogen deficient by the ISTH-BAT and EN-RBD-BSS. The sensitivity of the ISTH-BAT was 88% for adult men, 41% for adult women and 80% for children. The overall sensitivity of the EN-RBD-BSS to detect fibrinogen deficiency was 70%, i.e., 82% for adult men, 59% for adult women and 76% for children. When only afibrinogenemic patients were considered, the sensitivity of the ISTH-BAT in adult men, adult women and children was 100%, 54% and 61% and it was 87%, 100% and 61%, respectively for EN-RBD-BSS.

Bleeding severity

Only 6 of 100 (6%) investigated cases were asymptomatic, including 1 case with afibrinogenemia, 1 with hypofibrinogenemia and 4 with dysfibrinogenemia. Grade I, II and III bleeding severity were observed in 22 (22%), 29 (29%) and 43 (43%) patients, respectively (Figure 4). Overall, there was a moderately negative correlation between bleeding severity and Fg:C ($r = -0.468$, $P < 0.001$) and Fg:Ag level as well ($r = -0.474$, $P = 0.005$). When only afibrinogenemia and hypofibrinogenemia were considered, the correlation between bleeding severity and Fg:C was still moderate and statistically significant ($r = -0.45$, $p < 0.001$). Based on the previously established threshold of fibrinogen levels for bleeding severity grade [18], the severity of bleeding in 67% of patients was correctly identified, that 26% of patients had milder bleeding than expected and only 7% of patients had more severe bleeding than expected. Of note, although 3% of patients were expected to be asymptomatic, they were grade I (2%) and grade II (1%). The median (range) of Fg:C in asymptomatic cases was 40.5 mg/dl (9-94 mg/dl) and for grade I, II and III were 36 mg/dl (0-117 mg/dl), 40 mg/dl (0-130 mg/dl) and 0 mg/dl (0-90 mg/dl), respectively.

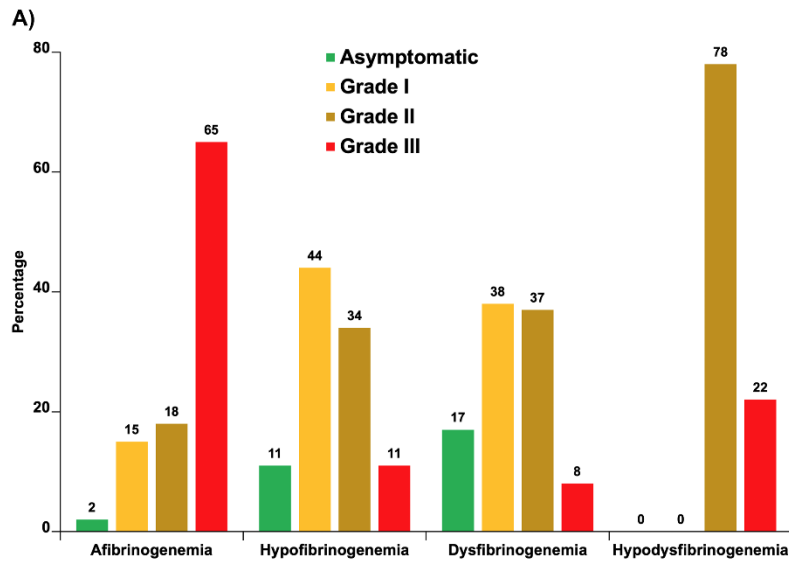
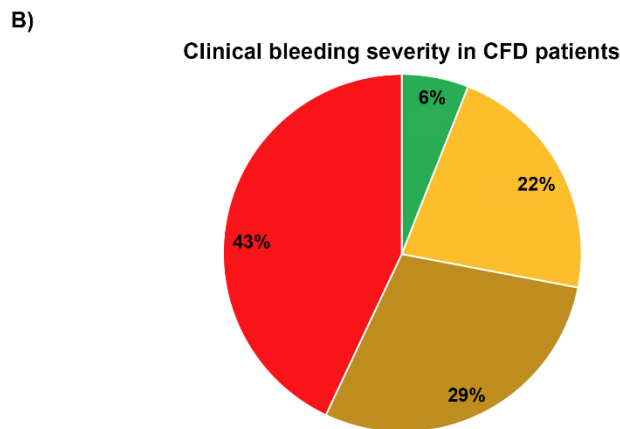


Figure 4. Frequency of the clinical bleeding severity grades in all study populations and in each group of patients.



Discussion

This study was conducted on the largest cohort of patients with fibrinogen deficiency so far evaluated for the ISTH-BAT and EN-RBD-BSS scores. Due to the rarity of fibrinogen deficiencies, collecting a large cohort of patients is challenging especially for patients with dysfibrinogenemia who usually present with mild clinical symptoms. In this study, we included 100 characterized cases with CFDs who have been evaluated for the BSSs and the bleeding severity. Compared to our cohort, Saes et al. found that Dutch patients diagnosed with CFDs ($n=41$) had a higher median ISTH-BAT (12 vs. 4). The lower bleeding score found in the present study may be explained by the fact that our cases were younger and assessed at the diagnosis age, therefore, probably experienced less hemostatic challenges (i.e., tooth extraction and surgeries). Indeed, we included 54 children (<18 years old), while Saes et al. reported 8 children only [17]. A difference in bleeding severity was also observed between the two cohorts, because we found a higher number of patients with grade III (43%), whereas Saes et al. found more patients with grade II bleeding severity (more than 50%) [17]. According to the established

baseline fibrinogen activity level [18], the bleeding severity grade recognized CFD correctly with a identified value of 67% in our study, in broad agreement with the Dutch cohort in which more than 60% of bleeding severity grade identified CFD accurately [17].

As compared to our previously described EN-RBD cohort, this study population showed a more severe bleeding pattern [18]. The proportion of cases with a grade III clinical severity was similar to that of the EN-RBD cohort (43% vs. 42.3%). However, the prevalence of grade I (22% vs. 19.2%) and grade II severity (29% vs. 3.8%) was higher in the present study with a smaller number of asymptomatic cases (6% vs. 34.6%) [18].

Fibrinogen level is among one of the few coagulation factors that was previously reported to be correlated with BS in affected patients [19]. In our study, a statistically significant correlation was found between the factor activity level and two score systems (Figure 3). These correlations are indeed comparable to previous reports [17, 19]. In a study by Palla et al. who investigated a large cohort of different RBDs (n= 492, fibrinogen deficiency, n= 43), a moderate negative correlation was observed between the EN-RBD-BSS and Fg:C ($r = -0.538$, $P < 0.001$), this correlation was strongest among the other factor deficiencies [19]. In the Dutch cohort of RBDs, a moderate correlation ($r = -0.683$) was seen between ISTH-BAT and factor activity [17]. In addition, we observed moderately negative but statistically significant correlations between Fg:Ag and both BAT scores. While the correlation between ISTH-BAT and factor activity level still remained moderate after excluding dysfibrinogenemia and hypodysfibrinogenemia, the correlation for the EN-RBD-BSS was weak yet significant.

In this study, as well as in the other two cohorts, the ISTH-BAT and EN-RBD-BSS were correlated and accurately detected patients with RBDs [17, 19]. Indeed, a moderate positive correlation was observed between the ISTH-BAT and EN-RBD-BSS ($r = 0.597$, $P < 0.001$) in our study, consistently with the Dutch ($r = 0.692$, $P < 0.001$) and the EN-RBD ($r = 0.681$, $P < 0.001$) cohorts. Collectively, these results suggest that in addition to the ISTH-BAT, the EN-RBD-BSS may be useful to detect patients with CFDs. For fibrinogen deficiencies, a moderate correlation is reported between the coagulant activity level and bleeding severity grades in an independent study [17, 18], similar to our observed correlation. The added advantage for the EN-RBD-BSS over that of the ISTH-BAT is that it is specifically developed for RBD patients and the questions are tailored for this subgroup of bleeding disorders, thus, could carry independent clinical value when the diagnosis of RBD is suspected.

Normal levels of Fg:Ag in patients with dysfibrinogenemia may dilute the assessments that depend on the antigen levels [22]. Although fibrinogen levels were not higher than the upper threshold, the

relationship between Fg:C and bleeding severity after removal of patients with dysfibrinogenemia did not differ from the overall population ($r = -0.402$, $P < 0.001$).

In terms of bleeding manifestations cutaneous and umbilical cord bleedings were particularly common in these patients. In females, menorrhagia and postpartum bleeding were the most common symptoms in agreement with other studies [17, 23]. However, the incidence of bleeding after surgery was lower than in the Dutch population [17]. It should also be noted that children and younger patients may face less bleeding challenges such as tooth extraction and post-operative [24]. Thus, since the median age of diagnosis of the current study population was low and more than half of the patients were children, the lower median BS compared to other studies is understandable.

The overall percentage of the patients that were correctly identified by the EN-RBD-BSS was higher than that of the ISTH-BAT, consistent with the previous study [17]. Adult women were more likely to be diagnosed with EN-RBD-BSS, whereas the percentages of adult men and children diagnosed with the ISTH-BAT score were higher. In our study, the overall detection sensitivity of both score systems was lower in adult women in comparison to men and children. This lower overall sensitivity might be due to the lower proportion of patients with severe fibrinogen deficiency (i.e. afibrinogenemia) among adult women compared to adult men and children. Nevertheless, in afibrinogenemic patients, the EN-RBD-BSS was able to detect all adult females, while the ISTH-BAT was able to detect all males. The results align with those of the Dutch cohort, in which the EN-RBD-BSS identified more women and the ISTH-BAT was able to identify more men [17] and more children were detected by the EN-RBD-BSS in their study [17]. One of the limitations of the study is the lack of healthy controls to be compared with the patients. The second limitation is that the scores assigned to women with menorrhagia and postpartum hemorrhage might have not been interpreted accurately because their electronic medical records were not available and data were based on the patient's description collected by an expert physician. In addition, there were a limited number of hypofibrinogenemic and hypodysfibrinogenemic patients. The strength of this study, however, is that it included the largest cohort of patients with CFDs being investigated for the ISTH-BAT and EN-RBD-BSS, with a larger group of children than in previous studies [17, 19].

In conclusion, by employing the two available BSSs, the ISTH-BAT and EN-RBD-BSS, on this large cohort of patients with CFDs, a moderate positive correlation was found between the two systems. Our results indicate that in addition to the ISTH-BAT, the EN-RBD-BSS is useful to identify CFD patients with a good level of sensitivity. Our results also show that, based on the previously

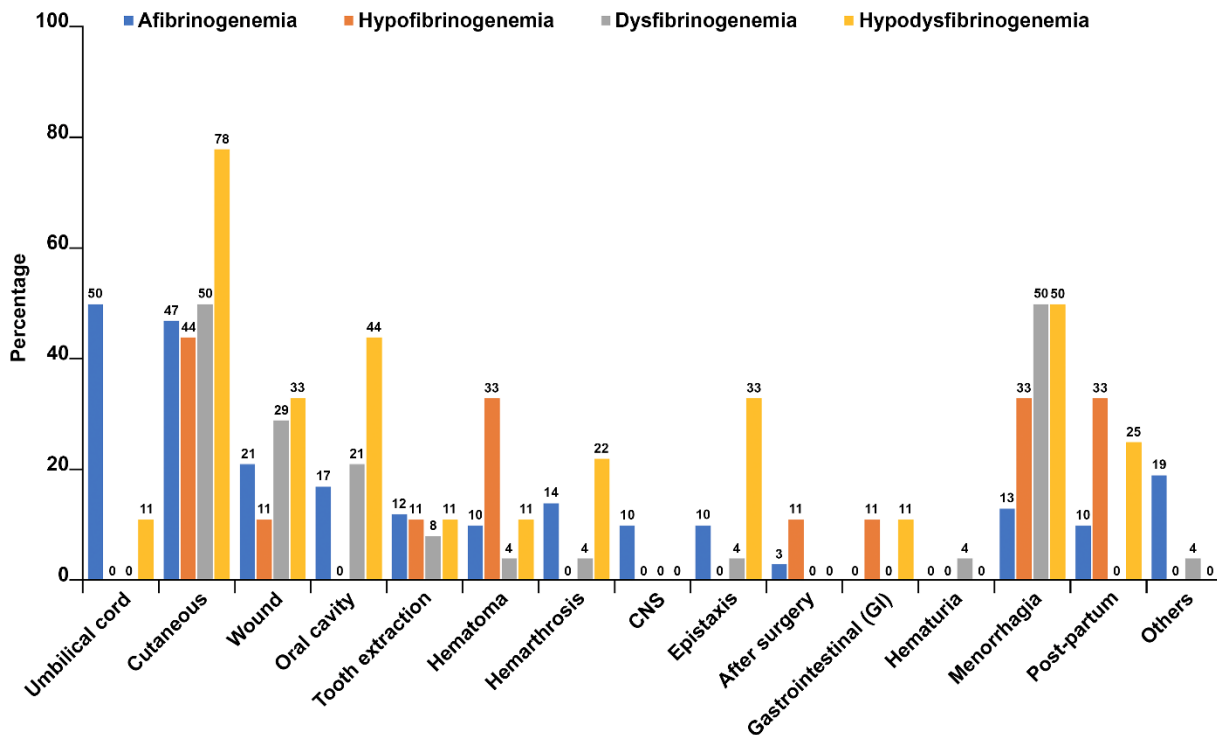
established baseline factor activity, the bleeding severity classification correctly identified the severity grades in almost two-thirds of patients.

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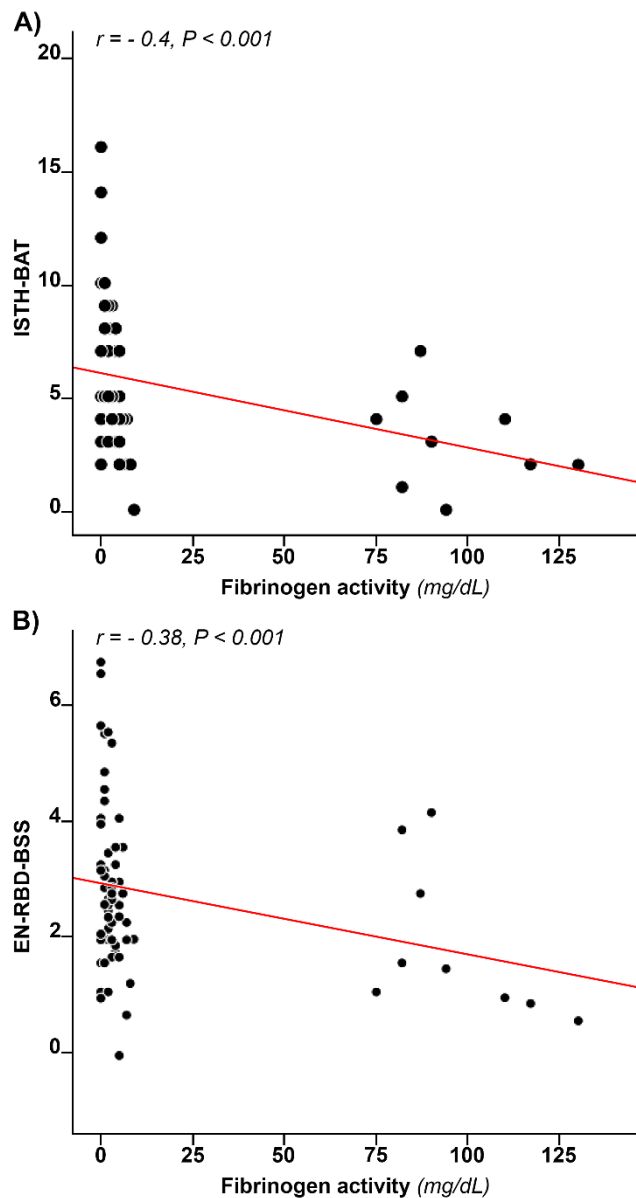
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Supplementary



Supplementary Figure 1. The reported frequency of various types of bleeding manifestations in different classifications of fibrinogen deficiencies. Other bleedings: circumcision (9 afibrinogenemic cases), ruptured ovarian cyst (2 afibrinogenemic cases), hyphema (1 dysfibrinogenemic case); CNS, Central Nervous System.



Supplementary Figure 2. Correlation between fibrinogen activity and bleeding assessment tools with linear regression lines. In patients with quantitative fibrinogen deficiencies (afibrinogenemia and hypofibrinogenemia), the correlation between Fg:C and the ISTH-BAT score (A) was moderately negative ($r = -0.4$, $P < 0.001$) and it was a weak but significant negative correlation between Fg:C and EN-RBD-BSS score ($r = -0.38$, $P < 0.001$, B).

Chapter III

Clinical and genetic investigation of patients with congenital fibrinogen disorders: A retrospective analysis of the PRO-RBDD

Samin Mohsenian^{1*}, Roberta Palla^{1*}, Marzia Menegatti², Andrea Cairo², Anna Lecchi², Alessandro Casini³, Marguerite Neerman-Arbez⁴, Rosanna Asselta^{5,6}, Sara Scardo², Simona Maria Siboni², Jan Blatny⁷, Ondrej Zapletal⁷, Jean-Francois Schved⁸, Muriel Giansily-Blaizot⁸, Susan Halimeh⁹, Mohamad Ayman Daoud⁹, Helen Platokouki¹⁰, Helen Pergantou¹⁰, Roger E.G. Schutgens¹¹, Monique Van Haaften-Spoor¹¹, Paul Brons¹², Britta Laros-van Gorkom¹³, Elise Van Pinxten¹³, Munira Borhany¹⁴, Naveena Fatima¹⁴, Danijela Mikovic¹⁵, Marko Saracevic¹⁵, Gül Nihal Özdemir¹⁶, Yilmaz Ay¹⁷, Michael Makris¹⁸, Caryl Lockley¹⁸, Andrew Mumford¹⁹, Andrew Harvey¹⁹, Steve Austin²⁰, Amy Shapiro²¹, Adrianna Williamson²¹, Catherine McGuinn²¹, Ilene Goldberg²², Philippe De Moerloose³, Flora Peyvandi^{1,2}

1. Università degli Studi di Milano, Department of Pathophysiology and Transplantation, Milan, Italy
2. Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center and Fondazione Luigi Villa, Milan, Italy
3. Division of Angiology and Hemostasis, Faculty of Medicine, Geneva University Hospitals, Geneva, Switzerland
4. Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Geneva, Switzerland
5. Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Milan, Italy
6. IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy
7. Department of Paediatrics Haematology and Biochemistry, University Hospital Brno and Masaryk University, Brno, Czech Republic
8. Department of Biological Hematology, CHU Montpellier, Université de Montpellier, Montpellier, France
9. Hematology Research Center GmbH, Duisburg, Germany
10. Haemophilia-Centre-Haemostasis Unit, Aghia Sophia Children's Hospital, Athens, Greece
11. Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
12. Department of Pediatric Hematology, Radboud University Medical Center, Nijmegen, The Netherlands
13. Department of hematology, Radboud University Medical Center, Nijmegen, The Netherlands
14. Clinical Hematology, National Institute of Blood Diseases and Bone Marrow Transplantation, Karachi, Pakistan
15. Blood Transfusion Institute of Serbia, Hemostasis Department, Belgrade, Serbia
16. Istinye University, Pediatric Hematology Department, Istanbul, Turkey
17. University of Health Sciences Kartal Health Application and Research center, Pediatric Hematology and Oncology Clinic, Istanbul, Türkiye

18. Sheffield Haemophilia and Thrombosis Centre, Royal Hallamshire Hospital, Sheffield, UK
19. School of Cellular and Molecular Medicine, University of Bristol, Bristol, UK
20. Department of Haematology, Guys and St Thomas' NHS Foundation Trust, London, UK
21. Indiana Hemophilia and Thrombosis Center, Indianapolis, IN, USA
22. Department of Pediatrics, Division of Pediatric Hematology Oncology, Weill Cornell Medicine, New York, USA

* **S.M** and **R.P** contributed equally to this manuscript.

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Abstract

Congenital fibrinogen deficiency (CFD) is a rare bleeding disorder caused by mutations in *FGA*, *FGB* and *FGG*. We sought to comprehensively characterize patients with CFD using PRO-RBDD (Prospective Rare Bleeding Disorders Database). Clinical phenotypes, laboratory and genetic features were investigated using retrospective data from the PRO-RBDD. Patients were classified from asymptomatic to grade III based on their bleeding severity. In addition, *FGA*, *FGB* and *FGG* were sequenced to find causative variants. A total of 166 CFD cases from 16 countries were included, of whom 123 (30 afibrinogenemia, 33 hypofibrinogenemia, 55 dysfibrinogenemia, 5 hypodysfibrinogenemia) were well characterized. Considering the previously established factor activity and antigen level thresholds, bleeding severity was correctly identified in 58% of cases. The rate of thrombotic events among afibrinogenemic and hypofibrinogenemic patients were relatively similar (11% and 10%) and surprisingly higher than in dysfibrinogenemic cases. The rate of spontaneous abortions among 68 pregnancies was 31%, including 86% in dysfibrinogenemic women and 14% with hypofibrinogenemia. 86 patients received treatment (69 on-demand and/or 17 on prophylaxis), fibrinogen concentrates being the most frequently used product. Genetic analysis was available for 91 cases and 41 distinct variants were identified. Hotspot variants (*FGG*, p.Arg301Cys/His and *FGA*, p.Arg35Cys/His) were present in 51% of dysfibrinogenemia. Obstetric complications were commonly observed in dysfibrinogenemia. This large multicenter study provided a comprehensive insight into the clinical, laboratory and genetics of patients with CFDs. We conclude that bleeding severity grades were in agreement with the established factor activity threshold in nearly half of the cases with quantitative defects.

Introduction

Coagulation proteins are the core components of the coagulation cascade, which leads to a complex interplay of reactions that prevent blood loss by converting soluble fibrinogen to insoluble fibrin strands through the interaction with platelets. Fibrinogen is a 340 KDa glycoprotein that is produced in the liver and circulates in blood at 150-450 mg/dl plasma concentrations [1]. This glycoprotein consists of two sets of three polypeptide chains, called alpha (α), beta (β), and gamma (γ), which are encoded by the *FGA*, *FGB* and *FGG* genes on chromosome 4 [2]. Congenital fibrinogen disorders (CFD), caused by mutations in these genes leading to fibrinogen deficiency and/or dysfunction [3], are classified as quantitative (type 1) or qualitative (type 2) deficiencies based on fibrinogen plasma levels. Quantitative deficiencies are characterized by the complete lack of fibrinogen in plasma (afibrinogenemia) or by a proportional decrease (hypofibrinogenemia) of fibrinogen coagulant activity (Fg:C) and antigen levels (Fg:Ag) (4). Qualitative deficiencies are described by normal Fg:Ag and decreased Fg:C (dysfibrinogenemia) or by a disproportional decrease of both Fg:C and Fg:Ag levels (hypodysfibrinogenemia) [4]. Afibrinogenemia, the rarest fibrinogen deficiency, has an estimated prevalence of one in 1 million but is more frequent in countries where consanguineous marriages are common and is often diagnosed in the newborn period [5, 6]. Patients with congenital afibrinogenemia show a severe bleeding symptoms, mostly manifesting in the neonatal period with umbilical cord bleeding or after circumcision [7]. Afibrinogenemic cases mainly experience bleeding but may also experience thrombotic events with or without replacement therapy, mainly because of the impairment of the anti-thrombin function of fibrin and related inability to sequester thrombin [8-10]. Inherited hypofibrinogenemia is associated with a mild bleeding tendency and more rarely by thrombotic complications [11]. Most patients with inherited dysfibrinogenemia are asymptomatic and are usually identified through abnormal blood coagulation tests or their family history. However, some patients may experience bleeding (rarely spontaneously) or thromboembolic complications due to preventing thrombin from binding to fibrin, thus leading to an increased level of free thrombin in the blood [12, 13]. Additionally, thrombosis can also occur when the fibrin clot strength, structure, or stability are altered [14]. Since fibrinogen plays a major role during pregnancy, women with CFD are at increased risk for adverse obstetric outcomes [11] related to the fact that fibrinogen plays an essential role in maintaining the placenta, supporting early-term trophoblast proliferation and spreading, and supporting the maternal-fetal circulation [15-17].

The severity of clinical manifestations is dependent on the fibrinogen activity plasma level. In a retrospective study from the European Network of Rare Bleeding Disorders (EN-RBD), including 46 patients with fibrinogen deficiency, patients with a mean fibrinogen activity level of at least 70 mg/dl

appeared to be protected from spontaneous bleeds, while a level >100 mg/dl completely protected the individual from any bleeding symptoms [18]. Because the clinical assessment of hemorrhagic symptoms is an important step in the evaluation of patients, bleeding assessment tools (ISTH-BAT and the EN-RBD-BSS) have been established to standardize the assessment of bleeding symptoms which can help to distinguish individuals affected by a bleeding tendency from those unaffected [19-22]. According to the study by the EN-RBD, RBD patients can be classified as asymptomatic, grade I, grade II, or grade III based on locations, potential clinical impact and spontaneity of bleeding [18]. In the same study, the established threshold of fibrinogen activity level necessary to prevent bleeding was above 100 mg/dL, and an undetectable fibrinogen level corresponds to the presence of major spontaneous bleeding (grade III) [18].

Genetic variants of *FGA*, *FGB* and *FGG* are useful for the correct diagnosis and to identify its types and subtypes as well [23]. More than 250 causative mutations have been reported in online databases associated with CFD [24, 25]. Heterozygous or homozygous mutations in the three fibrinogen genes can lead to congenital quantitative fibrinogen disorders [26]. Almost all cases of afibrinogenemia are due to null mutations (i.e. nonsense mutations, splice site mutations, frameshift mutations, large deletions) and are mostly located in *FGA*. In hypofibrinogenemia, heterozygous missense mutations are more frequent and distributed throughout the three genes. In most dysfibrinogenemic cases, the disease is caused by heterozygous missense mutations in *FGA* and *FGG*; hypodysfibrinogenemic patients are heterozygous or combined heterozygous, and their causative mutations are mainly located in *FGG* [27]. Despite the relative rarity of CFD, the number of cases reported so far is substantial [14, 25, 27]. Nonetheless, combining the results obtained from laboratory phenotype, genotype and clinical analyses is warranted to yield valuable information on the development and course of these diseases as well as for the choice of the appropriate treatment. Therefore, the establishment of international collaborations and registries are key steps that help to improve the patient management.

Considering this background, we performed a cross-sectional study within the Prospective Rare Bleeding Disorders Database (PRO-RBDD) with the goal to describe the laboratory, clinical and genetic features of a well-defined group of patients with CFD. In addition, we aimed to assess the association between factor activity level and clinical severity in a more comprehensive way to confirm the EN-RBD results in a wider and different cohort of patients [18]. Another aim was to find the difference between the age of diagnosis among different types of fibrinogen deficiency. This study was designed within the European Haemophilia Network (EUHANET) activities with the support of the European Association for Haemophilia and Allied Disorders (EAHAD) and the European

Commission Health Programme through the Executive Agency for Health and Consumers (EAHC) [28].

Methods

Data collection

This analysis was performed using data retrieved at baseline from the PRO-RBDD (demographic data and clinical historical data from the date of birth to the enrolment date), with a cross-sectional design [29]. A total of 166 records of patients with CFD were collected and entered into the database from February 2013 to 2020. Data were available on demographics (age at data collection and sex), fibrinogen coagulant activity and antigen levels (not at the time of treatment), lifelong clinical histories up to the date of inclusion into the study, age at first bleeding, age at diagnosis, reason for diagnosis, and types of treatment. Cases were evaluated in relation to clinical severity defined as asymptomatic (i.e., no documented bleeding episodes), grade I (i.e., bleeding after trauma or drug-induced), grade II (i.e., spontaneous minor bleeding), and grade III (i.e., spontaneous major bleeding) [18]. Data were collected from 36 centers in 16 countries, including Italy (n=38), the United Kingdom (n=14), the Netherlands (n=12), Pakistan (n=11), Greece (n=8), Turkey (n=7), the United States (n=6), Iran (n=5), Germany (n=5), France (n=4), Switzerland (n=4), Czech Republic (n=4), Colombia (n=2), Bosnia and Herzegovina (n=1), Serbia (n=1) and Indonesia (n=1). The project was approved by the Ethical Review Board of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy and written informed consent was obtained from all enrolled patients or their parents.

Laboratory assays

The historical diagnosis of fibrinogen deficiency was based on coagulant activity levels below the normal threshold of each laboratory; however, plasma samples and blood cells from most cases were shipped for diagnosis confirmation and genetic analysis to central laboratories (Laboratory of Molecular Pathology of the Angelo Bianchi Bonomi Haemophilia and Thrombosis Centre, Milan, Italy, and Division of Angiology and Haemostasis University Hospitals of Geneva, Geneva, Switzerland). Fg:C was measured by the Clauss method and Fg:Ag level by the turbidimetric latex immunoassay (HYPHEN BioMed, LIAPHEN, Neuville-sur-Oise, France). Most patients were classified based on the activity/antigen ratio that was used to distinguish between hypofibrinogenemia and dysfibrinogenemia (the ratio is $\text{Fg:C/Fg:Ag} < 0.7$ in dysfibrinogenemia and is $\text{Fg:C/Fg:Ag} > 0.7$ in hypofibrinogenemia) [30]. Half of the lowest detection limit was considered to represent a complete deficiency to facilitate analysis relating to factor activity levels (i.e., 5 mg/dl for a detection limit of 10 mg/dl in afibrinogenemic cases).

Genetic analysis

Genomic DNA was amplified by PCR (polymerase chain reaction) under standard conditions using sense and antisense primers designed on the basis of known sequences of the fibrinogen cluster. Direct sequencing of amplified fragments was performed by Sanger sequencing. The Human Gene Mutation Database (<https://www.hgmd.cf.ac.uk/ac/index.php>) and the Leiden Open Variation Database (<https://www.lovd.nl/3.0/home>) were used to find the already reported variants associated with CFD. Novel variants were evaluated using in silico prediction by Polymorphism Phenotyping (PolyPhen; <http://genetics.bwh.harvard.edu/pph2/>), Sorting Intolerant From Tolerant (SIFT; <http://provean.jcvi.org/>), Combined Annotation Dependent Depletion (CADD; <https://cadd.gs.washington.edu/>) and VarSome (<https://varsome.com/>) software classification and varSEAK (<https://varseak.bio/>) was used to predict splice site variants.

Statistical analysis

The Kruskal–Wallis test was used to compare: A) median ages at diagnosis between the type of fibrinogen deficiencies; B) median factor activity levels between the grade of bleeding severity, *P-value* < 0.05 being considered statistically significant. Pairwise comparisons between group levels with corrections for multiple testing were calculated (correction by Benjamini, Hochberg). Sensitivity, specificity and positive predictive value (precision) were evaluated for established factor activity thresholds in quantitative fibrinogen deficiency. Statistical analysis was performed using IBM SPSS Statistics 26 and R Studio version 4.0.3.

Results

Patient data

Out of the 166 cases recorded in the PRO-RBDD, 123 (80 adults, 43 children) had available results for both Fg:C and Fg:Ag, so that all analyses were performed on this population. The demographic data and laboratory results are shown in Table 1. Patients were classified as afibrinogenemia (n=30, 24%), hypofibrinogenemia (n=33, 27%), dysfibrinogenemia (n=55, 45%) and hypodysfibrinogenemia (n=5, 4%). The median (range) age of cases at study inclusion was 26 (0-85) years. The median age at diagnosis was significantly lower in afibrinogenemic patients, with a median age of 1 year (0-24), than in other groups while the highest was recorded in cases with dysfibrinogenemia (median 29, 0-84). After excluding cases with hypodysfibrinogenemia (because of insufficient data available for separate analyses, n=5) we confirm a statistically significant difference among the median ages at diagnosis of the three CFD subgroups (Figure 1A).

Table 1. Main features of the study population

Features	Afibrinogenemia (n=30)	Hypofibrinogenemia (n=33)	Dysfibrinogenemia (n=55)	Hypodysfibrinogenemia (n=5)	All cases (n=123)
Probands, n (%)	26 (86%)	23 (67%)	35 (64%)	3 (60%)	87 (71%)
Sex (M/F)	14/16	15/18	18/37	1/4	48/75
Age at inclusion (yrs) ¹	16 (1-47)	29 (0-65)	40 (4-85)	25 (4-29)	26 (0-85)
Age at diagnosis (yrs) ¹	1 (0-24)	18 (0-61)	29 (0-84)	23 (0-25)	16 (0-84)
Fg:C (mg/dl) ^{1, 2}	Undetectable	75 (30-141)	51 (21-152)	45 (41-52)	49 (0-152)
Fg:Ag (mg/dl) ^{1, 2}	Undetectable	88 (19-160)	277 (184-438)	1.17 (88-122)	128 (0-438)
Bleeding severity ³					
Asymptomatic	-	11 (34%)	22 (42%)	3 (60%)	36 (30%)
Grade I	1 (3%)	9 (28%)	8 (15%)	1 (20%)	19 (16%)
Grade II	7 (23%)	8 (25%)	19 (35%)	-	34 (29%)
Grade III	22 (73%)	4 (13%)	3 (6%)	1 (20%)	30 (25%)
Frequency of bleeding ³					
No bleeding	-	11 (34%)	22 (42%)	3 (60%)	36 (30%)
once a time	-	-	1 (2%)	-	1 (1%)
<1per year	3 (10%)	8 (25%)	6 (12%)	1 (20%)	18 (15%)
1 per year	10 (33%)	4 (13%)	10 (19%)	-	24 (20%)
2-3 per year	5 (17%)	5 (15%)	1 (2%)	-	11 (9%)
>3per year	12 (40%)	4 (13%)	12 (23%)	1 (20%)	29 (25%)
Thrombosis, Total ⁴	3 (11%)	3 (10%)	3 (6%)	-	9 (8%)
Venous	2 (66%)	1 (33%)	2 (66%)		5 (56%)
Arterial	-	1 (33%)	1 (33%)		2 (22%)
Both	1 (33%)	1 (33%)	-		2 (22%)
Treatment, Total ⁵	30 (100%)	20 (65%)	32 (63%)	4 (100%)	86 (70%)
On-demand	18 (60%)	19 (95%)	26 (81%)	4 (100%)	67 (78%)
Prophylaxis				-	
Both ⁶	7 (23%)	-	6 (19%)	-	13 (15%)
	5 (17%)	1 (5%)	-		6 (7%)

Bleeding treatment ⁷					
FC	16 (53%)	13 (65%)	14 (44%)	4 (100%)	47 (55%)
Cryo	13 (43%)	-	-	-	13 (15%)
FFP	10 (33%)	2 (10%)	1 (3%)	-	13 (15%)
Anti-fibrinolytic	7 (23%)	6 (30%)	16 (50%)	2 (50%)	31 (36%)
Blood	5 (17%)	1 (5%)	-	-	6 (7%)
Vit K	1 (3%)	-	-	-	1 (1%)
Other	-	1 (5%)	7 (22%)	-	8 (9%)
Reason for diagnosis					
Presented with bleeding, n (%)	30 (100)	20 (60%)	5 (11)	2 (40%)	57 (46%)
Screen for Kinship with proband, n (%)	1 (3%)	10 (30%)	17 (31%)	3 (60%)	31 (25%)
	-	3 (9%)	12 (24%)	1 (20%)	16 (13%)
Tested before surgery, n (%)	3 (12%)	10 (31%)	9 (18%)	1 (20%)	23 (19%)
Tested for other reasons, n (%)	2 (7%)	1 (3%)	4 (7%)	-	7 (6%)
Unknown reasons for testing, n (%)					

¹ The laboratory results and ages are reported as median (range)

² Normal range of plasma fibrinogen level is 150-350 mg/dl

³ The data regarding bleeding episodes were not available for 4 cases (1 hypofibrinogenemia, 3 dysfibrinogenemia)

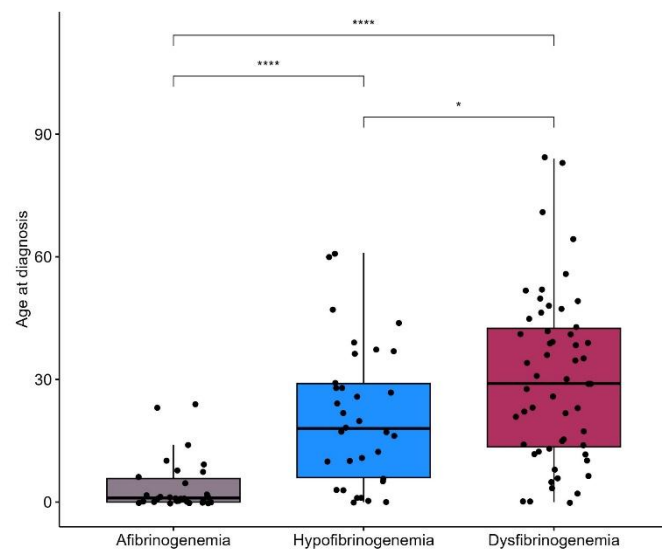
⁴ There are 11 missing data regarding thrombosis (2 afibrinogenemia, 3 hypofibrinogenemia, 5 dysfibrinogenemia, 1 hypodysfibrinogenemia)

⁵ There are 7 missing data for treatment (1 hypofibrinogenemia, 5 dysfibrinogenemia and 1 hypodysfibrinogenemia)

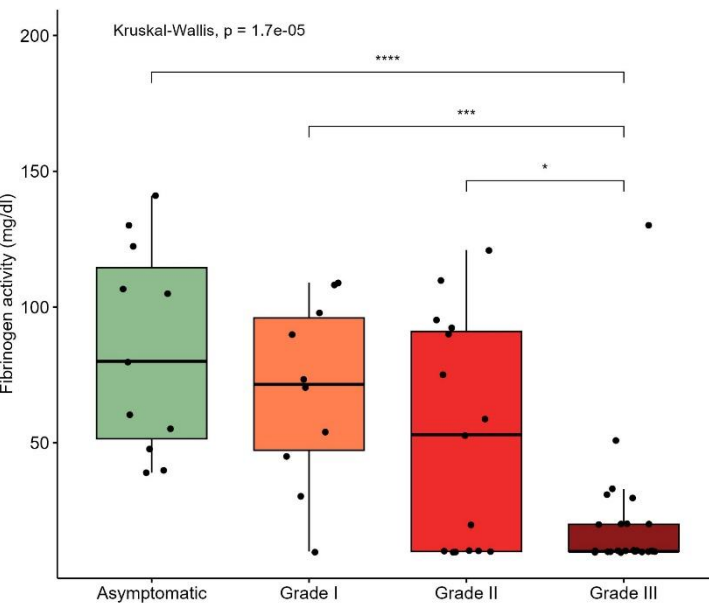
⁶ Patients who received both on demand and prophylaxis treatments during their lives

⁷ FC: Fibrinogen concentrate, FFP: fresh frozen plasma

A



B



C

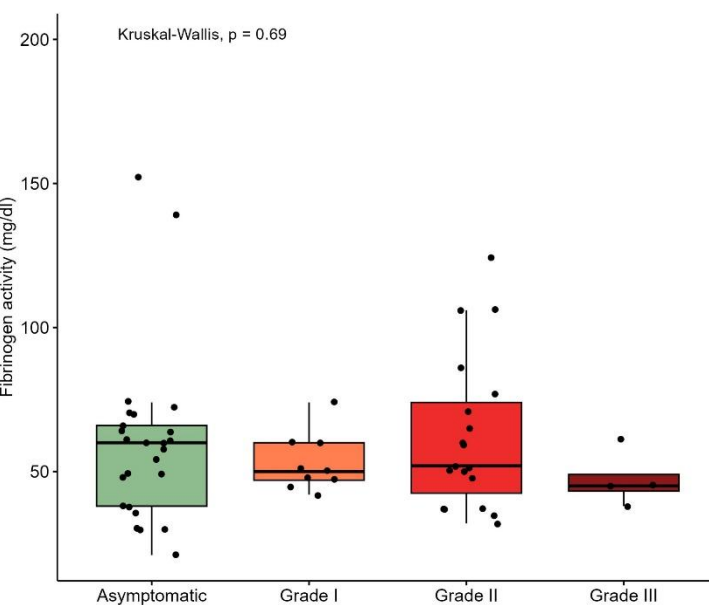


Figure 1. A) The box plot compares the median age at diagnosis in three different fibrinogen deficiency groups. The solid line indicates the median. B & C) The box plot compares the median factor activity levels between all grades of severity in quantitative (B) and qualitative (C) deficiencies. The Kruskal-Wallis test was performed to determine differences in the age at diagnosis between different subclasses of fibrinogen disorders. * (*P*-value < 0.05), ** (*P*-value < 0.01) and *** (*P*-value < 0.001).

Bleeding severity

More than 70% of afibrinogenemic patients had grade III bleeding, whereas the majority of those with dysfibrinogenemia and hypofibrinogenemia were asymptomatic (Table 1). An agreement of the bleeding severity grades with the established factor activity threshold in quantitative fibrinogen deficiencies was observed in 36 of 62 cases, i.e., with an accuracy of 58% (95% CI: 44.85-70.49). Taking the severity grades as a reference, 45% of those asymptomatic had factor activity level above 100 mg/dl, 56% of grade I-II cases had activity levels between 20 and 100 mg/dl. In patients with grade III bleeding, 65% had factor activity level below 20 mg/dl (Table 2). Except grade III in quantitative fibrinogen deficiencies, there was no significant differences between median levels of factor activity for other bleeding severity grades (Figure 1B).

Table 2. Determined factor activity (Clauss) thresholds (mg/dl) according to the bleeding severity grades in quantitative fibrinogen deficiencies.

Reference: Grades of severity	Factor activity levels	Grades of severity		
		Asymptomatic	Grade I & Grade II	Grade III
	Fg:C>100	5 (45%)	4 (16%)	1 (4%)
	20 ≤ Fg:C ≤ 100	6 (55%)	14 (56%)	8 (31%)
	Fg:C<20	0 (0%)	7 (28%)	17 (65%)
	Total	11	25	26

* There is a missing data regarding the grades of bleeding severity

Accordingly, in quantitative fibrinogen deficiencies the highest sensitivity was found for grade III (65%) followed by grade I-II (56%) and asymptomatic (45%), whereas the highest specificity (90%) was found for asymptomatic cases (Table 3). Additionally, we were interested in determining the

positive predictive value (precision) of established thresholds for different bleeding grades. Our analysis showed that the highest precision was observed in cases with Fg:C<20 mg/dl (71%) which means that 71% of patients with this value were correctly diagnosed as a grade III.

We further investigated the association between fibrinogen activity and bleeding severity in quantitative fibrinogen deficiencies (afibrinogenemia and hypofibrinogenemia), that resulted in a significant association (P -value <0.0001, Figure 1B), at variance with qualitative deficiencies (P -value = 0.69, Figure 1C).

Table 3. Specificity and sensitivity of bleeding severity grades and precision for the established factor activity threshold in quantitative fibrinogen deficiencies.

	Asymptomatic	Grade I & Grade II	Grade III
Sensitivity	45%	56%	65%
Specificity	90%	62%	81%
Positive predictive value (Precision)	50%	50%	71%

Reasons for diagnosis

All patients with afibrinogenemia and most of those with hypofibrinogenemia were identified due to bleeding episodes, while most of the patients with dysfibrinogenemia were diagnosed upon screening for surgery/other clinical question (42%) or as relatives of an affected case (31%), with only 11% due to bleeding (Table 1).

Clinical manifestations

A total of 71% of cases had bleeding episodes at least once in their lives. Figure 2 illustrates the bleeding manifestations in relationship to the different classifications of CFD. Cutaneous bleeding was the most common bleeding symptom (77%) in afibrinogenemic cases followed by hematoma (63%) and umbilical cord bleeding (48%). Of note, hematoma, central nervous system (CNS) bleeding and hemarthrosis were significantly more frequent in afibrinogenemic cases (63%, 33% and 30% respectively). Menorrhagia (36%, 33%) and post-partum hemorrhage (33%, 33%) were the most prevalent manifestations in dysfibrinogenemic and hypofibrinogenemic cases (Figure 2). Nine patients in our study experienced thrombosis (3 with afibrinogenemia, 3 hypofibrinogenemia and 3 dysfibrinogenemia) of which 56% were venous and 44% arterial.

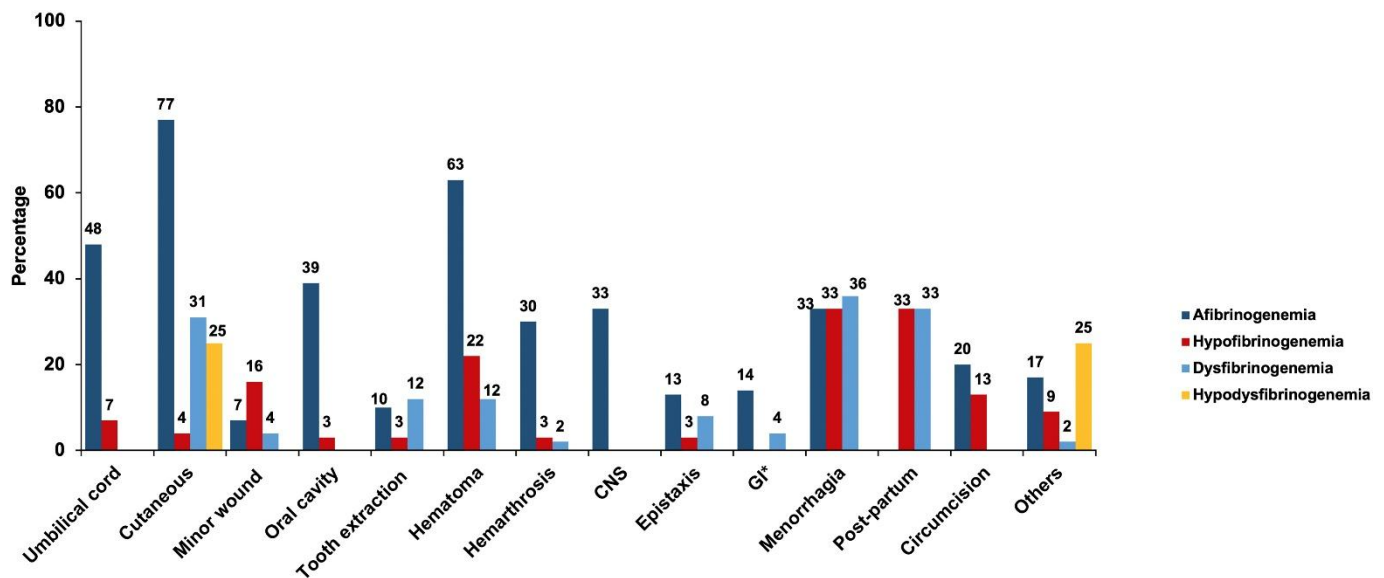


Figure 2. Reported frequency of different types of bleeding symptoms. Menorrhagia was considered in women who were in fertile age, and post-partum bleeding was assessed in women who underwent labor.

*Gastrointestinal bleeding

Pregnancies and complications

Of the 57 (46%) adult women included in this study, 32 (56%) had 68 pregnancies (1 afibrinogenemia, 16 hypofibrinogenemia, 49 dysfibrinogenemia and 2 hypodysfibrinogenemia) resulting in 47 (69%) live birth (1 afibrinogenemia, 13 hypofibrinogenemia, 31 dysfibrinogenemia and 2 hypodysfibrinogenemia). A high rate of spontaneous abortions occurred among adult women (21 in 68 pregnancies, 31%) including 18 (86%) in dysfibrinogenemia and 3 (14%) in hypofibrinogenemia. Regarding spontaneous abortions, two cases experienced three episodes, three cases two and nine cases one each with 14 out of 16 (87%) abortions occurring in the first-trimester of gestation (there were 5 missing information). Among 14 women who had a spontaneous abortion, 11 (78%) had at least one live birth. Post-partum hemorrhage was observed in 10 cases of which 70% were dysfibrinogenemic and 30% were hypofibrinogenemic. Two dysfibrinogenemic cases (10%) had bleeding during pregnancy.

Treatment

A total of 86 patients (56 females and 30 males) received treatment, including 69 on-demand and/or 17 with prophylaxis. Table 1 shows that all afibrinogenemic and hypodysfibrinogenemic cases and more than 60% of those dysfibrinogenemic and hypofibrinogenemic cases received treatment, most of them only on demand treatment (78%). Prophylaxis was reported in 40% of afibrinogenemic and in 19% of dysfibrinogenemic cases. The majority of cases on prophylaxis were living in Europe (13,

81%) and in the United States (2, 13%), and most were women (71%) and 59% of them had afibrinogenemia. Fibrinogen concentrate (FC) was the most frequent product used in afibrinogenemic (54%), hypofibrinogenemic (65%) and hypodysfibrinogenemic (100%) cases, while dysfibrinogenemic cases mainly received anti-fibrinolytic drugs (50%). Among afibrinogenemic patients on prophylaxis, 7 cases received FC, 4 cryoprecipitate and 4 FFP. Furthermore, all dysfibrinogenemic cases on prophylactic treatment were prescribed antifibrinolytic drugs.

Mutational spectrum

As a result of mutational screening using Sanger sequencing of *FGA*, *FGB*, and *FGG*, 41 causative variants were found in 91 patients (Table 4). Among them, three variants were novel including 1 in afibrinogenemia (c.115-610G>C, *FGB*), 1 in hypofibrinogenemia (p.Ser142Ter, *FGG*) and 1 in hypodysfibrinogenemia (p.Asp346His, *FGG*). Table 5 summarizes the potential pathogenic effect of the novel variants using the different in silico tools.

Table 4. Variants identified in the CFD population

Gene	cDNA	Mature chain	Exon	Intron	Type of variant	Observed Genotype	Patients (Probands/Relatives)	Classification	Bleeding/Thrombosis	Grade of severity	Novelty
FGA	c.95G>A	p.Gly32Glu	2		Missense	Hetero	3 (2/1)	Dysfibrinogenemia	3/0	0	-
FGA	c.103C>T	p.Arg35Cys	2		Missense	Hetero	1 (1/0)	Dysfibrinogenemia	0/0	0	-
FGA	c.104G>A	p.Arg35His	2		Missense	Hetero	6 (5/1)	Dysfibrinogenemia	3/0	0, I, II	-
FGA	c.112A>G	p.Arg38Gly	2		Missense	Hetero	6 (3/3)	Dysfibrinogenemia	6/0	0, I, II	-
FGA	c.114G>C	p.Arg38Ser	2		Missense	Hetero	1 (1/0)	Dysfibrinogenemia	1/0	II	-
FGA	c.163T>C	p.Cys55Arg	2		Missense	Hetero	1 (1/0)	Hypodysfibrinogenemia	0/0	0	-
FGA	c.180+1G>C	-		2	Splice site	Hetero	4 (1/3)	Hypofibrinogenemia/ Hypodysfibrinogenemia	3/1	0, I	-
FGA	c.196delT	p.Ser66LeufsTer5	3		Frameshift	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.385C>T	p.Arg129Ter	4		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.434T>A	p.Val145Asp	4		Missense	Hetero	5 (1/4)	Hypofibrinogenemia	5/0	I, II	-
FGA	c.448C>T	p.Gln150Ter	4		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.502C>T	p.Arg168Ter	4		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.510+1G>T	-	4		Splice site	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.532C>T	p.Arg178Ter	5		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.533_534insT	p.Ser179IlefsTer8	5		Frameshift	Homo	2 (1/1)	Afibrinogenemia	2/1	II, III	-
FGA	c.598C>T	p.Gln200Ter	5		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.711dupT	p.Lys238Ter	5		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.743G>A	p.Trp248Ter	5		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.934delA	p.Ser312AlafsTer109	5		Frameshift	Homo	4 (2/2)	Afibrinogenemia	4/0	II, III	-
FGA	c.945delT	p.Gly316GluTer105	5		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGA	c.1001G>A	p.Trp334Ter	4		Nonsense	Homo	3 (2/1)	Hypofibrinogenemia	3/0	I, III	-
FGA	c.1410dupT	p.Gly471TrpfsTer3	5		Frameshift	Hetero	3 (2/1)	Dysfibrinogenemia	3/0	0, II	-
FGA	c.1417G>A [§]	p.Asp473Asn	5		Missense	Compound hetero with c.1244+1G>T	1 (1/0)	Hypofibrinogenemia	0/1	0	-
FGB	c.115-610G>C	-		1	Deep intronic	Homo	1 (1/0)	Afibrinogenemia	1/0	III	✓
FGB	c.139C>T	p.Arg47Ter	2		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
FGB	c.490G>A	p.Asp164Asn	3		Missense	Hetero	2 (1/1)	Hypofibrinogenemia	2/1	I, II	-
FGB	c.1067A>G	p.Tyr356Cys	7		Missense	Homo	2 (1/1)	Afibrinogenemia	2/0	III	-
FGB	c.1148T>G	p.Leu383Arg	7		Missense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-

<i>FGB</i>	c.1244+1G>T [§]	-		7	Splice site	Compound hetero with c.1417G>A, Hetero	2 (2/0)	Hypofibrinogenemia	0/1	0	-
<i>FGG</i>	c.123+1G>A	-		2	Splice site	Homo	1 (1/0)	Afibrinogenemia	1/0	II	-
<i>FGG</i>	c.323C>G	p.Ala108Gly	4		Missense	Hetero	1 (1/0)	Hypofibrinogenemia	0/0	0	-
<i>FGG</i>	c.400C>T	p.Arg134Ter	4		Nonsense	Homo	1 (1/0)	Afibrinogenemia	1/0	III	-
<i>FGG</i>	c.425C>A	p.Ser142Ter	5		Nonsense	Hetero	1 (1/0)	Hypofibrinogenemia	0/0	0	✓
<i>FGG</i>	c.571G>A	p.Gly191Arg	6		Missense	Hetero	2 (1/1)	Hypofibrinogenemia	2/0	I	-
<i>FGG</i>	c.901C>T	p.Arg301Cys	8		Missense	Hetero	10 (5/5)	Dysfibrinogenemia	6/2	0, I, II, III	-
<i>FGG</i>	c.902G>A	p.Arg301His	8		Missense	Hetero	3 (2/1)	Dysfibrinogenemia	3/0	I, II	-
<i>FGG</i>	c.998A>G	p.His333Arg	8		Missense	Hetero	2 (1/1)	Hypofibrinogenemia	0/0	I	-
<i>FGG</i>	c.1024G>A	p.Asp342Asn	8		Missense	Hetero	3 (2/1)	Dysfibrinogenemia/ Hypofibrinogenemia	2/0	0, II	-
<i>FGG</i>	c.1036G>C	p.Asp346His	8		Missense	Hetero	1 (1/0)	Hypodysfibrinogenemia	1/0	I	✓
<i>FGG</i>	c.1151C>G	p.Ser384Cys	9		Missense	Hetero	5 (2/3)	Dysfibrinogenemia	3/0	0, I, II	-
<i>FGG</i>	c.1201C>T	p.Arg401Trp	9		Missense	Hetero	2 (2/0)	Hypofibrinogenemia	1/0	0, I	-

Table 5. In silico prediction of novel variants in the CFD cohort

Novel variants	Protein	Gene	Exon/ Intron	Classification	Fg:C/Fg:Ag (mg/dl)	Bleeding severity	In silico prediction	
							Scores	Varsome
c.1036G>C	p.Asp346His	<i>FGG</i>	Ex 8	Hypodysfibrinogenemia	45/117	Grade I	CADD:28, SIFT:0 PolyPhen:1	Likely pathogenic
c.425C>A	p.Ser142Ter	<i>FGG</i>	Ex 5	Hypofibrinogenemia	105/109	Asymptomatic	CADD:36	Likely pathogenic
c.115-610G>C	-	<i>FGB</i>	Int 1	Afibrinogenemia	undetectable	Grade III	varSEAK:1, CADD:6.9	Not classified

- Four different Insilco tools and VarSome were used to predict the effects of novel variants. These tools were including Polymorphism Phenotypingv2 (PolyPhen-2), Sorting Intolerant from Tolerant (SIFT) and Combined Annotation Dependent Depletion (CADD). Splice variants were assessed using the varSEAK software and the CADD score. As we expected, nonsense variants were also predicted to be harmful using the CADD score.

- Polyphen score: >0.908: probably damaging; >0.446 and ≤0.908: possibly damaging; ≤0.446: benign.

- SIFT score: <0.05: deleterious; ≥0.05: tolerated.

- CADD score: >20, deleterious
- varSEAK Classification: Class 1: No splicing effect, Class 2: Likely no splicing effect, Class 3: Unknown splicing effect, Class 4: Likely splicing effect, Class 5: Splicing effect

In afibrinogenemia and hypofibrinogenemia:

In 23 afibrinogenemic patients 18 different variants were identified of which 13 (72%) were null mutations (i.e. nonsense and frameshift), 2 (11%) missense, 2 splice site (11%), and 1 (6%) intronic (Figure 3A). Of note, all cases with afibrinogenemia were homozygous. Mutations were found in *FGA*, *FGB* and *FGG* in 66%, 22% and 12% of cases and exon 5 *FGA* was a hotspot that embedded 39% of mutations.

Out of 25 hypofibrinogenemic patients with available genetic results, 20 (80%) cases were heterozygous, 3 (12%) were homozygous, and only one case was compound heterozygous for two variants. Among 12 different causative variants, missense mutations were the most common (67%), even though 2 (16%) null mutations were also identified. Six (50%) variants were located on *FGG*.

In dysfibrinogenemia and hypodysfibrinogenemia:

All cases with dysfibrinogenemia (n=39) were heterozygous for a missense mutation in *FGA* and *FGG* except for 3 cases in whom a heterozygous frameshift variant was identified in *FGA*: p.Gly471TrpfsTer3. There were 10 different identified variants in dysfibrinogenemia, all located in *FGA* (exon 2, 50%), and *FGG* (exon 8, 30%). The majority of variants (90%) were missense; however, one frameshift variant (10%) was also found. Almost 51% of identified variants in dysfibrinogenemia were hot spot mutations located at residues Arg301 in exon 8 of *FGG*, (n=13, 34%), and at Arg35 in exon 2 of *FGA* (n=7, 18%). Of note, the p.Arg38Gly, *FGA* variant was also frequent, being identified in 6 cases (15%). In hypodysfibrinogenemic cases (n=4) three different variants were identified, two of them in exon 2 and intron 2 of *FGA*, and one in exon 8 of *FGG*. Two hypodysfibrinogenemic cases carried heterozygous missense variants (p.Cys55Arg, *FGA* and p.Asp346His, *FGG*) and remaining 2 cases heterozygous splice site variants, c.180+1G>C, *FGA* (Table 4).

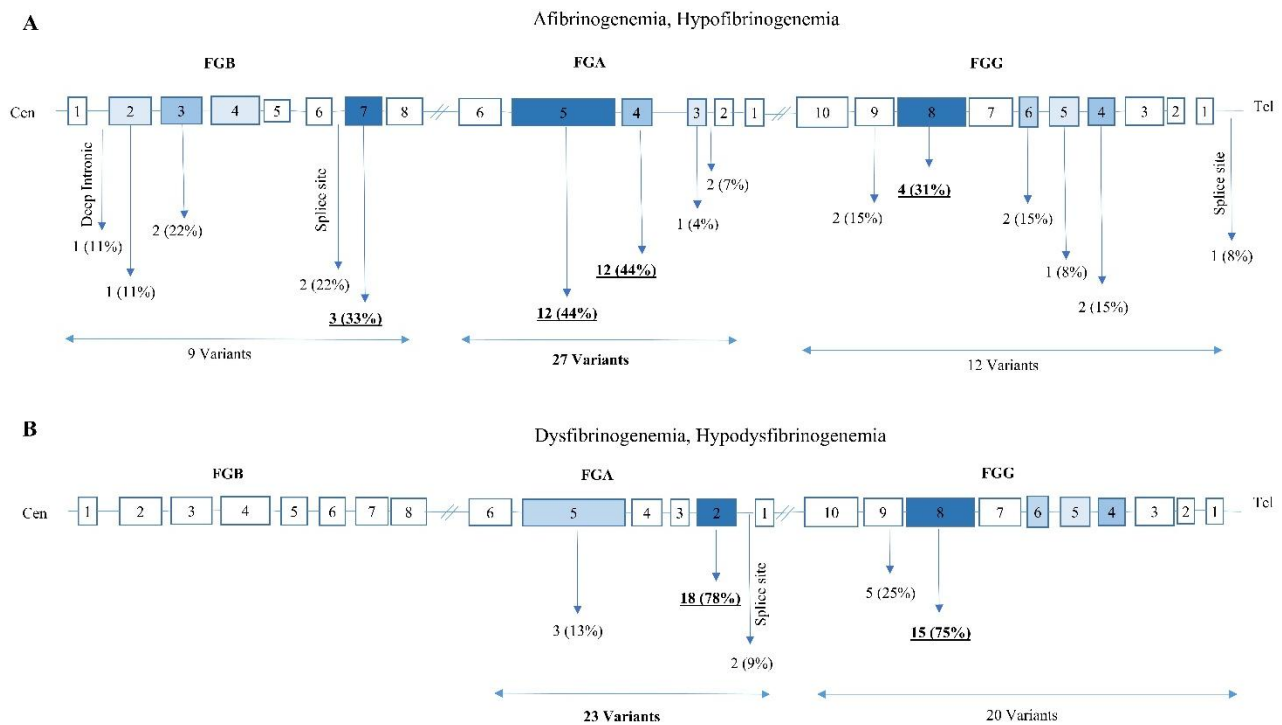


Figure 3. Distribution of causative variants in (A) quantitative and (B) qualitative fibrinogen deficiencies

Among the 21 cases who experienced spontaneous abortions, 6 had genetic results. Three of them (50%) carried the p.Arg301Cys, *FGG* mutation and two of them had two spontaneous abortions and one case had one. A woman with this variant had a live birth. The p.Arg301Cys variant was identified in two of nine patients (22%) who developed thrombosis.

Discussion

This multi-centric study is one of the most comprehensive ever conducted to describe the bleeding frequency, treatment products, complications and genetics stemming from an international large cohort of patients with CFD. According to our results, the bleeding severity of nearly half of patients was correctly identified on the basis of the established threshold of Fg:C [18]. In both previous Iranian and Dutch studies, the established fibrinogen activity level correctly identified the bleeding severity in more than 60% of cases in agreement with our results (58%) [31, 32]. Given that the sensitivity of the bleeding severity grades was low for asymptomatic and grade I-II (45% and 56% respectively), further studies with larger cohorts are warranted to establish factor activity level thresholds. Compared to the other rare bleeding disorders, there is a significant association between bleeding severity and Fg:C levels in quantitative fibrinogen deficiency [18] which is indeed confirmed by the present findings ($P\text{-value} < 0.0001$). In contrast, no strong association was found in qualitative

deficiencies (*P-value* = 0.69, Figure 1C) which could indicate that other underlying variables contributed to the clinical bleeding phenotype in this group.

In our cohort, there was a significant difference between the age at diagnosis among CFD classifications, indicating that afibrinogenemic patients were diagnosed at a younger age (median 1), but those with dysfibrinogenemia were principally diagnosed in adulthood (median 29). The difference between median ages among CFD groups can show that patients with afibrinogenemia often experienced severe bleeding which led to diagnosis in early ages. Early diagnosis and adequate treatment can prevent life-threatening bleeding in afibrinogenemia. In contrast, hypo- or dysfibrinogenemia typically occurs in older adults because of their good prognosis. Regarding bleeding manifestations, the most common symptom in our afibrinogenemic patients was cutaneous bleeding (77%), which was close to the results reported by Sumitha et al (65%) [33]. However, in contrast to another study, umbilical cord bleeding was not the most common bleeding symptom (85% vs 48%) [34]. In addition, in our recent study, the incidence rate of umbilical cord bleeding and cutaneous bleeding was almost the same (47%) [27]. Due to the wide variety of bleeding manifestations in patients with CFD, it is difficult to determine which symptoms are the most frequent [27, 35]. Nonetheless, women with hypofibrinogenemia and dysfibrinogenemia are more prone to suffer from obstetric complications [36]. Of note, these complications are more frequent in afibrinogenemic patients when treatment products are not available. A third of our women experienced spontaneous abortions, which was within the reported range (20% to 50%) for hypofibrinogenemia and dysfibrinogenemia [14, 36].

Thrombosis events occur less frequently in hypofibrinogenemic patients than in afibrinogenemic patients. The reduced amount of circulating fibrinogen in hypofibrinogenemia is thought to reduce the risk of thrombosis, which is more prevalent in afibrinogenemia [37, 38, 39]. In the absence of fibrin, the anti-thrombin action of fibrin is reduced, which could increase circulating thrombin levels and cause thrombosis [39, 40]. Although thrombosis prevalence has been more frequently reported in qualitative fibrinogen deficiencies [41], the thrombotic events were surprisingly similar among our afibrinogenemic and hypofibrinogenemic cases and higher than in dysfibrinogenemic cases. These findings are comparable with those of a recent study by Hugon-Rodin et al [42]. It is unclear whether replacement therapy can lead to thrombotic events, because some studies have reported thrombosis is associated with replacement therapies in CFD, but others believe that treatment entails a low risk of thrombosis for CFD patients [8, 40, 43-45]. In the current study, among 19 patients who received prophylaxis, there was only one afibrinogenemic case (5%) who developed thromboembolism after fibrinogen concentrate.

Even though 73% of the afibrinogenemic patients were severe, only 40% received prophylaxis. This can be explained by the fact that despite the important efforts to better assess the epidemiology and molecular mechanisms leading to CFD, data on clinical management are still scarce. Indeed, owing to the rarity of these diseases and the absence of randomized controlled studies, recommendations, and guidelines are mainly based on expert consensus rather than on evidence-based data. Another explanation for the avoidance to prescribe prophylaxis may be a limited access to fibrinogen products in countries with high rates of afibrinogenemia.

A total of 41 different gene variants were identified in this cohort. In afibrinogenemia, the majority of disease-causing variants were nonsense (72%) followed by frameshift (11%) and missense (11%) variants. The identification of c.115-600A>G variant in the *FGB* intron 1 was a cause of afibrinogenemia as already reported by Davis et al., demonstrating the value of screening deep intronic regions [46]. Accordingly, following a screening of this region, we identified a novel c.115-610G>C variant in an afibrinogenemic case with grade III bleeding.

The novel p.Asp346His, *FGG* variant was found in a hypodysfibrinogenemic case with mild bleeding symptoms. Using the UniProt database, this variant was found to impair fibrin polymerization by altering calcium binding [47]. In the present study, a 36-year-old hypofibrinogenemic patient with compound heterozygous mutations (c.1417G>A, *FGA* and c.1244+1G>T, *FGB*) developed thrombosis but never bled. Nevertheless, c.1244+1G>T, *FGB* was reported in an afibrinogenemic case with spontaneous bleeding [25].

In dysfibrinogenemic cases, hotspot variants (p.Arg301Cys/His, *FGG* and p.Arg35Cys/His, *FGA*) were identified in 51% them in agreement with previous reports [25]. Most patients with fibrinogen deficiency who carry the variants in the Arg301 residue do not experience any thrombotic event. It is suggested that the combination of a factor V Leiden defect and the p.Arg301Cys may predispose to thromboembolism [48]. In a study in which 52 dysfibrinogenemic cases had variants in Arg301, *FGG*, 26% and 27% of p.Arg301His and p.Arg301Cys carriers developed thrombosis, indicating no significant association with the risk of thrombosis or major bleeding was reported [49]. We found a tendency to both bleeding and thrombosis in 20% of the cases with p.Arg301Cys, *FGG*, although all the three cases with p.Arg301His, *FGG* had only bleeding. Thus, there is no conclusive evidence that the genotype of these variants contributes to the thrombosis phenotype. We had six cases with dysfibrinogenemia carrying p.Arg35His who demonstrated clinical manifestations ranging from asymptomatic to grade II. In other studies, cases with this variant showed bleeding as well as thrombosis [14, 50]. For example, Castaman et al. reported a patient with menorrhagia who had an

ischemic stroke at older age [50]. In contrast, those cases were asymptomatic in another study [25]. Altogether, these results indicate the difficulty of treating CFD due to its heterogeneous clinical manifestations. The only case carrying p.Arg35Cys, *FGA* was asymptomatic in contrast with the previous study by Casini et al [25]. Regarding the Arg38, *FGA* residue, even though the p.Arg38Gly and p.Arg38Ser variants have been reported to be associated with bleeding [51-53], in the current study 3 out of 7 (43%) dysfibrinogenemic cases were asymptomatic. For those women who experienced spontaneous abortions, genetic results were available for 6 cases in whom the variants in two residues were recurrent (p.Arg301Cys, *FGG* in 50%, p.Arg38Ser/Gly, *FGA* in 33%). Similarly, women who suffered post-partum hemorrhage all carried p.Arg301Cys/His, *FGG* or p.Arg38Gly, *FGA*. Overall, among 10 women with obstetrical complications (spontaneous abortion, post-partum hemorrhage and bleeding during pregnancy), 5 cases (50%) were carriers of p.Arg301Cys/His, *FGG*, 40% were carrier of p.Arg38Ser/Gly, *FGA* and only one case was carrier of p.Ser384Cys, *FGG*.

One of the limitations of our study concerns the lack of detailed data regarding thrombosis cases, the outcomes of treatments and the results of the ISTH-BAT scores. Owing to referral bias it is reasonable to suspect that not all patients have been entered into the database, so that the present population might be enriched by patients with more severe symptoms. Also, there were a limited number of hypodysfibrinogenemic cases. In addition, we have limited data to know whether patients who experienced bleeding episodes were on treatment or were all patients studied before any treatment. It is worth noting that the effects of potential common variants that may change the fibrinogen level and severity cannot be neglected. On the other hand, the strength of this study lies in the international collaborative network which created a web database with a large population to comprehensively collect the clinical phenotype and genotype data of patients with CFD, even though addressing these research questions requires larger cohorts

In conclusion, this large multicenter study provided a comprehensive insight into the clinical, laboratory and genetics of patients with CFDs. The findings confirm that the bleeding severity characterizations developed by EN-RBDD [18] were concordant with the established factor activity threshold in nearly half of the patients with quantitative defects. The rate of thrombosis following prophylaxis with replacement therapy in the present study was 5%, but further studies are needed. Furthermore, obstetric complications are commonly observed in dysfibrinogenemic patients.

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Chapter IV

Gynecologic and obstetric complications in women with congenital fibrinogen disorders: insights from the Prospective Rare Bleeding Disorders Database

Samin Mohsenian¹, Roberta Palla¹, Marzia Menegatti², Andrea Cairo², Simona Maria Siboni², Marguerite Neerman-Arbez³, Mehran Karimi^{4,5}, Helen Pargantou⁶, Rosanna Asselta^{7,8}, Danijela Mikovic⁹, Marko Saracevic⁹, Britta Laros-van Gorkom¹⁰, Laura Jacobs¹⁰, Amy Shapiro¹¹, Adrianna Williamson¹², Michael Makris¹³, Alessandro Casini¹⁴, Flora Peyvandi^{1,2}

1. Università degli Studi di Milano, Department of Pathophysiology and Transplantation, Milan, Italy
2. Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center and Fondazione Luigi Villa, Milan, Italy
3. Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Geneva, Switzerland
4. Pediatric Hematology-Oncology Department, American Hospital Dubai, Dubai, United Arab Emirates
5. Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
6. Haemophilia Centre-Haemostasis and Thrombosis Unit, Aghia Sophia Children's Hospital, Athens, Greece
7. Department of Biomedical Sciences, Pieve Emanuele, Humanitas University, Milan, Italy
8. IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy
9. Hemostasis Department, Blood Transfusion Institute of Serbia, Belgrade, Serbia
10. Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands
11. Indiana Hemophilia and Thrombosis Center, Indianapolis, IN
12. Innovative Hematology, Inc. (Indiana Hemophilia & Thrombosis Center)
13. Sheffield Haemophilia and Thrombosis Centre, Royal Hallamshire Hospital, Sheffield, United Kingdom
14. Division of Angiology and Hemostasis, Faculty of Medicine, Geneva University Hospitals, Geneva, Switzerland

Abstract

Background

Women and girls with congenital fibrinogen deficiencies (CFDs) face higher hemorrhagic risks during their reproductive years, yet data on gynecological and obstetric complications remain limited.

Aim

We aimed to rate the prevalence of heavy menstrual bleeding (HMB) and obstetric complications in women with CFDs and compare our findings with previous reports.

Material and methods

This study analyzed data from the Prospective Rare Bleeding Disorders Database (PRO-RBDD) registry, including available fibrinogen activity and antigen levels, as well as clinical phenotype and genotype (2013-2020).

Results

A total of 59 women (8 afibrinogenemic, 15 hypofibrinogenemic, and 36 dysfibrinogenemic cases) were investigated of which 32 cases had 70 pregnancies. The prevalence of HMB was comparable between hypofibrinogenemic (27%) and dysfibrinogenemic (36%) cases, with a higher frequency in afibrinogenemia (75%). The rates of postpartum hemorrhage (PPH) at 36% and miscarriage at 23% were notably higher than those observed in the general population (1-10% and 10-20%). These complications were similarly distributed among dysfibrinogenemia (35%, 37%) and hypofibrinogenemia (36%, 31%). There were only 2 (4%) bleeds during pregnancy, both occurring in dysfibrinogenemia. Miscarriage was also observed in 50% of the asymptomatic dysfibrinogenemic cases. No significant difference in miscarriage and PPH rates was found between dysfibrinogenemic individuals with and without hotspot variants ($P=0.94$).

Conclusion

The high rate of obstetric complications in CFDs highlights the need for early diagnosis and the potential need for prophylaxis, as pregnancy may also pose risks in asymptomatic cases. Hotspot variants do not appear to increase the risk of obstetric complications.

Background

Congenital fibrinogen deficiencies (CFDs) are inherited bleeding disorders resulting from variants in the fibrinogen genes *FGA*, *FGB*, and *FGG* [1]. The disorder is categorized into quantitative (afibrinogenemia and hypofibrinogenemia) and qualitative (dysfibrinogenemia and hypodysfibrinogenemia) defects based on plasma fibrinogen levels [2]. Afibrinogenemia is characterized by the near-complete absence of fibrinogen in plasma, whereas hypofibrinogenemia involves a proportional reduction in both fibrinogen coagulant activity and antigen (Fibrinogen activity/antigen > 0.7). Dysfibrinogenemia, in contrast, is defined by reduced fibrinogen activity despite normal antigen levels, while hypodysfibrinogenemia is characterized by disproportionate reductions in both fibrinogen activity and antigen (Fibrinogen activity/antigen < 0.7) [2, 3]. Depending on factor plasma levels or genetic makeup, cases with CFDs exhibit a wide range of clinical symptoms, ranging from severe bleeding to being entirely asymptomatic or even experiencing thrombotic events [4, 5].

Women and girls with CFDs may experience more adverse outcomes given their physiological exposure to hemorrhagic risk during the reproductive age. Heavy menstrual bleeding (HMB) is often the most common symptom and in some cases the sole clinical manifestation of CFDs [6, 7]. Pregnancy poses additional challenges for affected women, because fibrinogen is a critical protein required for maintaining the placenta, supporting early trophoblast proliferation and spreading, thereby contributing to successful pregnancies [8, 9]. A previous studies indicate that women with afibrinogenemia and dysfibrinogenemia face a heightened risk of placental pathologies, such as abruption [10]. In contrast, in the Fibrinogest study, placental abruption (especially early in pregnancy) was more common in women with hypofibrinogenemia [11]. Additionally, the risk of miscarriage and bleeding during pregnancy is well-documented in those with severe CFDs [12]. Due to the high rate of early miscarriage in women with afibrinogenemia and severe hypofibrinogenemia, a fibrinogen concentrate should be administrated soon after pregnancy confirmation and must be continued during pregnancy to maintain a fibrinogen plasma level at ≥ 100 mg/dL [13, 14]. Women with severe or moderate hypofibrinogenemia or dysfibrinogenemia particularly those carrying prothrombotic variants, encounter a significantly increased risk of miscarriage due to low or dysfunctional fibrinogen. The prevalence of miscarriage in this population is reported to range from 20% to 50% [5, 15, 16], which is notably higher than the worldwide risk of miscarriage which is approximately 15.3% [17]. Women and girls with CFDs are at increased risk of bleeding due to their physiological exposure to hemorrhagic challenges during menstruation, pregnancy, childbirth and in the postpartum period. Despite these potential complications, data on gynecological and obstetric

outcomes in individuals with CFDs remain scarce. Understanding the impact of these disorders on pregnancy, miscarriage risk, and PPH is crucial for improving clinical management and developing preventive strategies.

Hence, in this study, we aimed to assess the rate of gynecological and obstetric complications in women and girls with CFDs using the Prospective Rare Bleeding Disorders Database (PRO-RBDD). This database compiled demographic and clinical data from birth to enrollment, providing insights into the medical history of affected individuals [7, 18].

Material and methods

This study analyzed baseline data recorded in the PRO-RBDD [7], encompassing 59 females in reproductive age (≥ 12 years old) with available fibrinogen activity and antigen levels, as well as clinical manifestation and genotype information, collected between 2013 and 2020. We also included women above the typical fertile age (e.g., >50 years), as they may have experienced relevant gynecological complications prior to the age at inclusion. The historical diagnosis of fibrinogen deficiency was based on coagulant activity levels below the normal threshold (reference range: 160-450 mg/dL). Fibrinogen activity levels were measured by the Clauss method and the antigen levels by the turbidimetric latex immunoassay (HYPHEN BioMed, LIAPHEN, Neuville-sur-Oise, France). For molecular analysis, samples were sent to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico) or to the Department of Genetic Medicine and Development in Geneva and direct sequencing of amplified fragments was performed by Sanger sequencing. Genetic variants were classified according to the ACMG guidelines established by the American College of Medical Genetics and Genomics [19]. Data were collected from centers across eight countries, primarily in Europe (Table 1). The severity of bleeding was evaluated according to the criteria of the European network of rare bleeding disorders (EN-RBD) [20]. HMB was defined as blood loss exceeding 80 mL per cycle, with variation possible depending on each center's protocols [21]. PPH was defined as an estimated blood loss greater than 500 mL after vaginal delivery or over 1000 mL following cesarean delivery [22]. The project was approved by the ethical review board of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy and written informed consent was obtained from all enrolled patients or their parents.

Statistical analysis

The chi-square test was used to compare the rate of menorrhagia and obstetric complications across different groups of CFDs. The Mann-Whitney test was applied to compare baseline factor activity

levels between livebirth and complicated pregnancies. Due to the small sample size, we performed Fisher's Exact Test to assess the potential association between cases carrying hotspot variants and those without. The analyses were conducted using RStudio 2022.7.1 and Excel 2016.

Results

Population

In this study we included 59 adult females (≥ 12 years old) consisting of 8 afibrinogenemia, 15 hypofibrinogenemia and 36 dysfibrinogenemia. Among them, 33 women (56%) had a total of 71 pregnancies, including 1 pregnancy in afibrinogenemia, 16 pregnancies in hypofibrinogenemia and 54 in dysfibrinogenemia. The only recorded pregnancy in an afibrinogenemic case resulted in miscarriage and was not managed with prophylactic therapy. Given the relatively low median age of these patients in this study (21 years, range: 12-47 years), the number of pregnancies observed was limited. This small number of pregnancies led to the exclusion of pregnancy outcomes in afibrinogenemia from further analysis. Therefore, we chose to describe obstetric complications between hypofibrinogenemia and dysfibrinogenemia (women, $n=32$, pregnancies, $n=70$) (Figure 1-A).

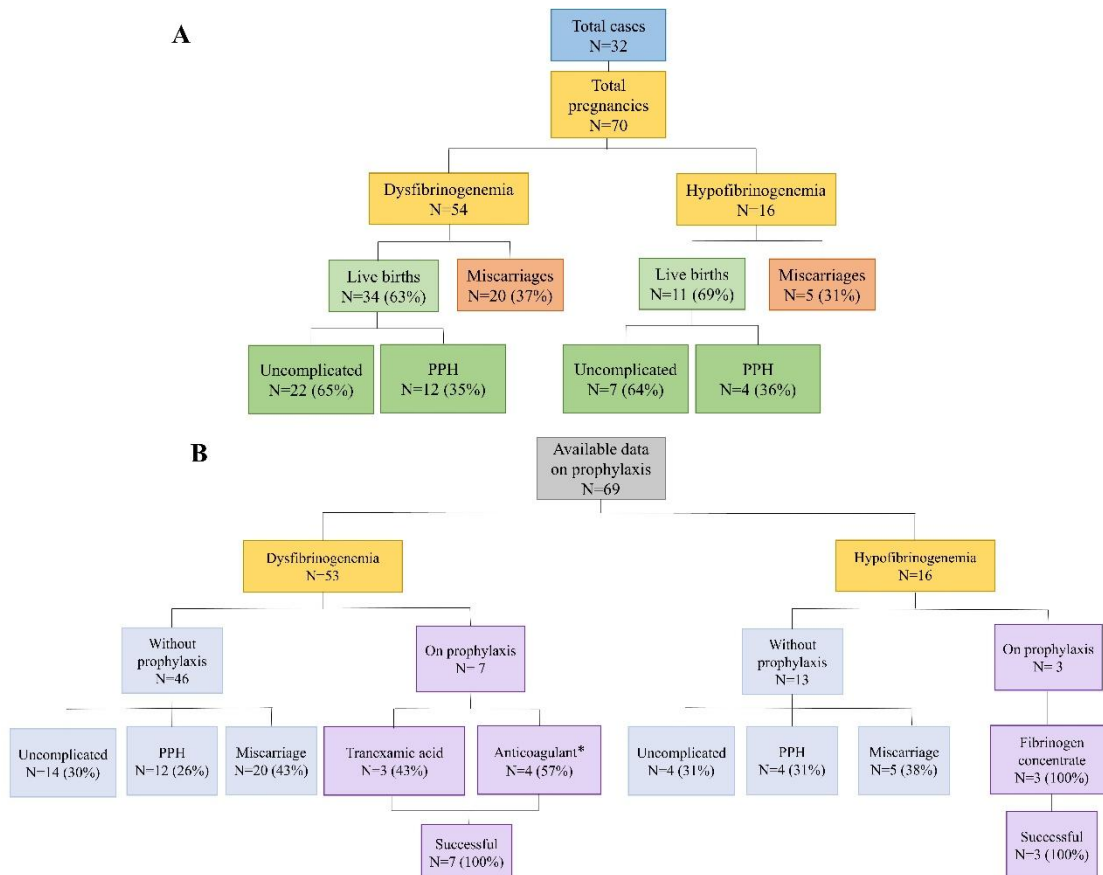


Figure 1. Flowchart of study population selection, including total pregnancies and prophylaxis status. **A)** Flowchart of pregnancy outcomes in patients with dysfibrinogenemia and hypofibrinogenemia. The outcomes are further divided into live births, PPH and miscarriages. **B)** Comparison of pregnancy outcomes between cases managed with prophylaxis and those without prophylaxis. *Four pregnancies were on thromboprophylaxis (unfractionated and low molecular weight heparin) because of previous miscarriages. PPH; Postpartum hemorrhage.

Fibrinogen activity levels were assayed at the time of inclusion with fibrinogen activity levels below 10 mg/dL in afibrinogenemia, median factor activity levels of 90 mg/dL (range 33-130 mg/dL) in hypofibrinogenemia and 60 mg/dL (range: 30-150 mg/dL) in dysfibrinogenemia. The median fibrinogen antigen levels in hypofibrinogenemia and dysfibrinogenemia were 118 mg/dL (range: 27-155 mg/dL) and 264 mg/dL (range: 184-438 mg/dL), respectively. Considering women with at least one pregnancy, genetic data were available for 15 dysfibrinogenemic and 5 hypofibrinogenemic cases.

Heavy menstrual bleeding

Of the 59 analyzed women, 21 (36%) experienced HMB. Among the 8 afibrinogenemic cases, 4 (50%) were on prophylaxis. Of those who did not receive prophylaxis, 3 (75%) experienced HMB. However, there is no significant difference between the proportions of HMB in hypofibrinogenemic (4 of 15, 27%), and dysfibrinogenemic (13 of 36, 36%) cases ($P=0.78$).

Obstetric complications

Considering all cases with at least one pregnancy, a total of 23 dysfibrinogenemic cases with 54 pregnancies and 9 hypofibrinogenemic cases with 16 pregnancies were analyzed (Figure 1-A). Among the 70 pregnancies, 45 (64%) resulted in live births, of which 16 (36%) were complicated by PPH, and none were on prophylaxis. Meanwhile, 25 (36%) pregnancies ended in miscarriage with recurrent pregnancy loss observed in 6 (38%) cases, accounting for 60% of all miscarriages (Table 1). Of note, among the available data on the timing of miscarriages, 8 out of 10 cases (80%) experienced at least one miscarriage during the first trimester of pregnancy.

In the 54 pregnancies of individuals with dysfibrinogenemia, 34 (63%) resulted in live births, with 22 (65%) being uncomplicated and 12 (35%) complicated by PPH. Additionally, 20 (37%) pregnancies ended in miscarriage.

Similarly, among the 16 pregnancies in individuals with hypofibrinogenemia, 11 (69%) resulted in live birth, with 7 (64%) being uncomplicated and 4 (36%) complicated by PPH. Moreover, 5

pregnancies (31%) resulted in miscarriage. When considering the severity of hypofibrinogenemia based on fibrinogen activity levels—severe (<50 mg/dL), moderate (50–100 mg/dL), and mild (>100 mg/dL up to the lower limit of the laboratory reference range)—only one severe case was reported, in which both pregnancies resulted in successful deliveries, possibly due to prophylactic fibrinogen replacement therapy. In moderate and mild cases, live birth rates were 67% (n=6) and 60% (n=3), respectively, while miscarriage occurred in 33% (n=3) and 40% (n=2) of cases. We found no significant difference in the rates of PPH and miscarriage between cases with dysfibrinogenemia and hypofibrinogenemia ($P=0.66$).

Among the 23 dysfibrinogenemic cases, 5 of them with a total of 10 pregnancies were asymptomatic before pregnancy. Of these, 5 pregnancies (50%) resulted in uncomplicated live births (cases, n=3), while the remaining 5 (50%) resulted in miscarriage (cases, n=3). Notably, one individual experienced both successful live births and complicated pregnancies.

Table 1. Obstetrical complications and characteristics of women who experienced pregnancy.

Case	Disorder	Age at inclusion	Pregnancy (N)	Miscarriage (N)	Miscarriage trimester	Live births	Fibrinogen activity (mg/dL) ^{1*}	cDNA	Protein	Gene	Transcript	Genotype ²	Exon/ Intron	ACMG ³ classification	Country
1	Dysfibrinogenemia	32	1	1	NA ³	0	152	c.1410dupT	p.Gly471TrpfsTer3	FGA	Frameshift	Hetero	5	Likely pathogenic	Italy
2	Dysfibrinogenemia	49	2	1	NA	1	35	c.114G>C	p.Arg38Ser	FGA	Missense	Hetero	2	Uncertain significance	Italy
3	Dysfibrinogenemia	52	1	0	NA	1	66	c.901C>T	p.Arg301Cys	FGG	Missense	Hetero	9	Pathogenic	Italy
4	Dysfibrinogenemia	41	1	0	NA	1	71	c.902G>A	p.Arg301His	FGG	Missense	Hetero	8	Pathogenic	Italy
5	Dysfibrinogenemia	67	2	0	NA	2	124	c.1410dupT	p.Gly471TrpfsTer3	FGA	Frameshift	Hetero	5	Likely pathogenic	Italy
6	Dysfibrinogenemia	25	3	1	After first trimester	2	106	NA	NA	NA	NA	NA	NA	NA	US
7	Dysfibrinogenemia	25	1	1	NA	0	61	NA	NA	NA	NA	NA	NA	NA	US
8	Dysfibrinogenemia	73	1	0	NA	1	74	c.902G>A	p.Arg301His	FGG	Missense	Hetero	8	Pathogenic	Italy
9	Dysfibrinogenemia	28	6	2	Before first trimester	4	91	NA	NA	NA	NA	NA	NA	NA	US
10	Dysfibrinogenemia	49	3	0	NA	3	52	c.112A>G	p.Arg38Gly	FGA	Missense	Hetero	2	Pathogenic	Italy
11	Dysfibrinogenemia	44	1	0	NA	1	77	c.112A>G	p.Arg38Gly	FGA	Missense	Hetero	2	Pathogenic	Italy
12	Dysfibrinogenemia	39	2	0	NA	2	139	NA	NA	NA	NA	NA	NA	NA	Italy
13	Dysfibrinogenemia	50	3	1	Before first trimester	2	42	c.1151C>G	p.Ser384Cys	FGG	Missense	Hetero	9	Uncertain significance	Italy
14	Dysfibrinogenemia	61	1	0	NA	1	48	NA	NA	NA	NA	NA	NA	NA	UK
15	Dysfibrinogenemia	76	4	1	After first trimester	3	45	c.901C>T	p.Arg301Cys	FGG	Missense	Hetero	8	Pathogenic	UK
16	Dysfibrinogenemia	49	4	2	Before first trimester	2	48	c.901C>T	p.Arg301Cys	FGG	Missense	Hetero	8	Pathogenic	Italy
17	Dysfibrinogenemia	28	1	0	NA	1	37	NA	NA	NA	NA	NA	NA	NA	UK
18	Dysfibrinogenemia	40	5	3	Before first trimester	2	60	NA	NA	NA	NA	NA	NA	NA	Switzerland
19	Dysfibrinogenemia	39	2	1	NA	1	50	NA	NA	NA	NA	NA	NA	NA	Switzerland
20	Dysfibrinogenemia	36	2	0	NA	2	86	c.902G>A	p.Arg301His	FGG	Missense	Hetero	8	Pathogenic	Italy
21	Dysfibrinogenemia	46	4	2	NA	2	51	c.901C>T	p.Arg301Cys	FGG	Missense	Hetero	8	Pathogenic	Italy
22	Dysfibrinogenemia	51	1	1	NA	0	72	c.112A>G	p.Arg38Gly	FGA	Missense	Hetero	2	Pathogenic	Italy
23	Dysfibrinogenemia	29	3	3	Before first trimester	0	60	NA	NA	NA	NA	NA	NA	NA	Serbia
24	Hypofibrinogenemia	30	2	0	NA	2	33	c.1001G>A	p.Trp334Ter	FGA	Nonsense	Homozygous	4	Likely pathogenic	Netherlands
25	Hypofibrinogenemia	38	1	0	NA	1	90	c.490G>A	p.Asp164Asn	FGB	Nonsense	Hetero	3	Likely pathogenic	Netherlands
26	Hypofibrinogenemia	26	2	0	NA	2	73	c.180+1G>C	-	FGA	Splice site	Hetero	Int2	Uncertain significance	Netherlands
27	Hypofibrinogenemia	61	1	0	NA	1	107	c.1024G>A	p.Asp342Asn	FGG	Missense	Hetero	8	Likely pathogenic	Italy
28	Hypofibrinogenemia	37	1	0	NA	1	90	c.998A>G	p.His333Arg	FGG	Missense	Hetero	8	Likely pathogenic	Greece
29	Hypofibrinogenemia	22	4	3	Before first trimester	1	75	NA	NA	NA	NA	NA	NA	NA	Iran

30	Hypofibrinogenemia	38	2	1	Before first trimester	1	130	c.709T>C	p.Tyr237His	FGG	Missense	Hetero	7	Likely pathogenic	Switzerland
31	Hypofibrinogenemia	37	1	0	NA	1	51	NA	NA	NA	NA	NA	NA	NA	Italy
32	Hypofibrinogenemia	36	2	1	Before first trimester	1	110	NA	NA	NA	NA	NA	NA	NA	Italy

¹ Fibrinogen activity reference range: 160-450 mg/dL. The fibrinogen activity levels were recorded at the time of inclusion.

² Homo, Homozygous. Hetero, Heterozygous

³ N.A. Not available

* Factor activity levels were measured using the Clauss method.

[‡] American College of Medical Genetics and Genomics. The ACMG classification is a standardized framework for interpreting genetic variants, particularly in relation to their potential to cause disease.

Genetic variants and pregnancy complications

Of the 32 women who experienced pregnancies, genetic data were available for 22 (69%), covering a total of 41 pregnancies; 32 in dysfibrinogenemia and 9 in hypofibrinogenemia. In dysfibrinogenemic cases, 9 of 16 (56%) carried hotspot variants (p.Arg301Cys/His) in the *FGG*, exon 8 (Table 1). Among their 19 pregnancies, 14 (74%) resulted in live births; 6 (43%) of these pregnancies were uncomplicated, while 8 (57%) were complicated with PPH. Notably, both hotspot variants are classified as pathogenic according to ACMG guidelines. The remaining 5 (29%) pregnancies resulted in miscarriage. Nevertheless, there is no statistically significant difference in the rates of miscarriage and PPH between individuals with and without hotspot variants ($P=0.94$).

Fibrinogen replacement prophylaxis and thromboprophylaxis

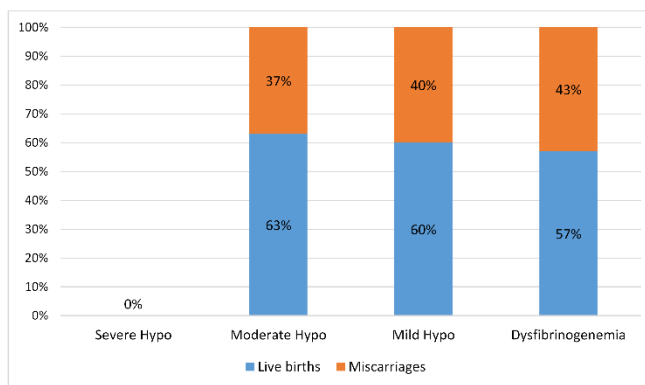
Among 31 cases with available prophylaxis data, 10 pregnancies received prophylaxis, all resulting in successful outcomes (Figure 1B). Among seven treated pregnancies in dysfibrinogenemic cases, three received tranexamic acid, while the remaining four were managed solely with antithrombotic prophylaxis (unfractionated heparin and low molecular weight heparin) after previous recurrent miscarriages. All pregnancies proceeded without PPH. All three pregnancies in women with hypofibrinogenemia were treated with fibrinogen concentrate, likely due to a history of bleeding diathesis, including hematoma, intermenstrual vaginal bleeding, and bleeding after tooth extraction.

In contrast, of the 59 pregnancies (46 in dysfibrinogenemia and 13 in hypofibrinogenemia) not managed with prophylaxis, 34 (58%) resulted in live births, of which 18 (53%) were without

complications, 16 (47%) were complicated by PPH and 25 (42%) of all pregnancies ended in miscarriage, with 75% occurring in the first trimester (Figure 2A, B).

Among 53 pregnancies in cases of dysfibrinogenemia, 46 were not managed with prophylaxis. Of 26 live births deliveries, 14 (54%) had a successful outcome, 12 (46%) resulted in PPH and 20 (43%) pregnancies ended in miscarriage (Figure 1B). Similarly, among 16 pregnancies in hypofibrinogenemic cases, 13 were not managed with prophylaxis. Among 8 livebirths, 4 (50%) had a successful outcome, 4 (50%) resulted in PPH and 5 (38%) pregnancies ended in miscarriage (Figure 2A, B).

A)



B)

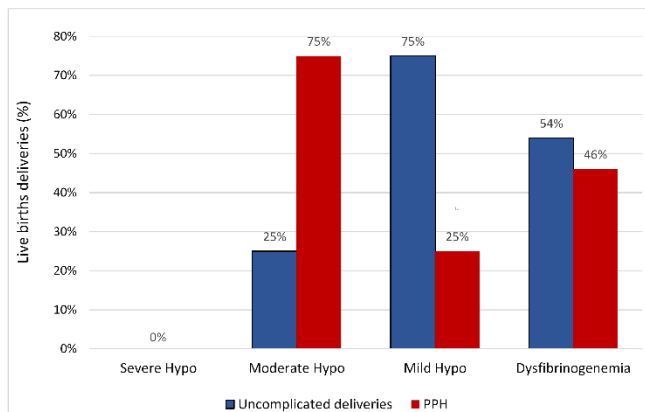


Figure 2. Comparison of pregnancy outcomes

between dysfibrinogenemic and hypofibrinogenemic cases without prophylaxis.

A) The chart shows the percentage of livebirths and miscarriages for each condition. The severity of hypofibrinogenemia is based on the fibrinogen activity level (severe: <50 mg/dL; moderate: 50–100 mg/dL; mild: >100 mg/dL to the lower range of laboratory). **B)** Considering live births in cases who were not on prophylaxis, the percentages of uncomplicated deliveries and those complicated by PPH were compared between dysfibrinogenemia and hypofibrinogenemia. There was one case of severe hypofibrinogenemia with two successful deliveries, both of which occurred under prophylaxis. PPH; Postpartum hemorrhage.

Discussion

Women with inherited bleeding disorders are at an increased risk of HMB and pregnancy-related bleeding; however, data on obstetric complications in cases of CFDs remain limited. This study, as part of the PRO-RBDD cohort, investigated HMB and obstetric complications in CFDs, with a particular focus on dysfibrinogenemia and hypofibrinogenemia. As afibrinogenemic cases are generally more severe and typically diagnosed at an early age, the cases included in this study also

had a relatively low median age of 21 years, which may have contributed to the limited number of pregnancies observed.

The rate of HMB did not differ significantly between dysfibrinogenemic and hypofibrinogenemic cases, with reported rates of 36% and 27%, respectively. Similarly to our previous study, HMB was identified as the most prevalent symptom in CFDs cases, with a prevalence of 33% [23]. However, the rate of HMB in afibrinogenemic cases who did not receive prophylaxis was 75%. Similarly, in both the RBiN and QualyAfib studies, HMB was reported in over 50% and 73.7% of the CFD population, respectively [24, 25]. This suggests that the occurrence of HMB in individuals with afibrinogenemia, particularly those are not on prophylactic treatment, may be a common and significant clinical feature across different patient cohorts.

The percentage of live birth and PPH in our study were 64% and 36%, respectively, which are almost comparable to the findings of a previous study by Hugon-Rodin et al. (74% and 20%) and notably higher than the PPH rate in the general population (1–10%) [11, 26]. Consistent with findings from this study, the prevalence of live births in cases of dysfibrinogenemia (63%) and hypofibrinogenemia (69%) was comparable (76.2 vs 71.1%) [11]. However, live births were slightly more frequent in cases of hypofibrinogenemia.

Among the total deliveries in dysfibrinogenemia, 22% resulted in PPH, with none of these cases receiving treatment. This rate is similar to that observed in two previous studies, which reported PPH rates of 21% and 20.7% [5, 11]. In the Fibrinogest study, the rate of PPH in women with hypofibrinogenemia was reported at 18.2%, which was lower than the rate observed in our study [11]. However, considering livebirths, the comparable rates of PPH for dysfibrinogenemia (35%) and hypofibrinogenemia (36%) highlight that both conditions pose substantial risks during pregnancy, emphasizing the need for vigilant monitoring and timely intervention. Notably, bleeding during pregnancy occurred exclusively in dysfibrinogenemic cases albeit with a low frequency (n=2, 4%), and none of these cases were on thromboprophylaxis.

In this study, the rate of miscarriage (36%) was higher than those observed in both the European and North American general populations (10%–20%) and the Fibrinogest study (13%) [11, 27, 28]. However, it was lower than that reported by Haverkate et al. (47%), likely due to the inclusion of patients with thrombotic-related fibrinogen variants in their cohort [15]. In a previous study by Casini et al., the miscarriage rate in dysfibrinogenemia was 19.8% based on 111 pregnancies [5]. Our study observed a higher rate of 37%, possibly due to the inclusion of more severe cases with greater obstetric complications. According to a few documented case reports, hypofibrinogenemia has been

associated with recurrent miscarriages, even though at a lower rate compared to other CFDs [29]. Nevertheless, the higher rate observed in this study (31%) underscores the critical need for careful management of pregnancies in affected women.

Interestingly, pregnancies in asymptomatic cases with dysfibrinogenemia were associated with miscarriage (50%). This suggests that pregnancy can lead to complications even in asymptomatic individuals with dysfibrinogenemia. Therefore, asymptomatic cases with dysfibrinogenemia should also be considered for routine follow-up and may benefit from prophylactic fibrinogen replacement therapy during pregnancy.

Baseline factor activity levels showed no significant difference between CFD women with live births and those with miscarriages, nor between those who experienced postpartum hemorrhage and those who did not ($P=0.98$ and $P=0.69$, respectively). Due to the limited sample size, these results were not included in the results. Therefore, further studies focused specifically on a large number of hypofibrinogenemia could provide valuable insights into the role of factor activity in predisposing individuals to obstetric complications.

The hotspot variants p.Arg301His/Cys in the *FGG* were identified in 56% of dysfibrinogenemic cases. Although these cases experienced obstetric complications, with 57% suffering from PPH and 29% of pregnancies resulting in miscarriage, no significant difference was found between cases with and without hotspot variants ($P=0.94$). Similarly, in a cohort of 101 dysfibrinogenemia cases, hotspot variants were not found to be associated with pregnancy complications [5].

The findings from this study showed the critical importance of prophylactic measures in improving pregnancy outcomes for women with CFDs. Since only 39% of those who were not on prophylaxis achieved successful outcomes, this demonstrates the considerable risks associated with inadequate management of CFDs during pregnancy. The high rates of miscarriage (42%) and PPH (27%) in the non-prophylaxis group further emphasize the vulnerability of these patients. Interestingly, the absence of a significant difference in outcomes between hypofibrinogenemic and dysfibrinogenemic cases without prophylaxis suggests that both conditions present comparable risks when left untreated. Therefore, although the rate of live births in CFDs was high (64%), these pregnancies were still associated with complications. This highlights the potential benefit of prophylactic measures in preventing severe outcomes, taking into account both family and the patient's history of bleeding or thrombotic events.

The management of dysfibrinogenemia during pregnancy poses unique challenges due to the risk of both bleeding and thrombosis [30]. Our dysfibrinogenemic case, who had successful deliveries with thromboprophylaxis, suggests that careful selection and considering of anticoagulant therapy can contribute to positive pregnancy outcomes in women with dysfibrinogenemia, particularly those with a history of recurrent miscarriages. However, due to the rarity and variability of this condition, a tailored approach should be based on a thorough multidisciplinary evaluation, with family history playing a key role in guiding treatment decisions.

This study had several limitations, including incomplete data on prophylaxis, the timing of miscarriages, lack of information on the type of delivery, and the absence of genetic results for some cases. Additionally, the low sample size limited our ability to assess the association between fibrinogen concentration and the risk of obstetric complications. This also hindered our ability to determine threshold levels required to prevent bleeding and miscarriages. Nevertheless, maintaining fibrinogen activity levels above 100 mg/dL during pregnancy and above 150 mg/dL during labor is recommended [13]. In addition, there may be a potential bias due to the inclusion of a higher proportion of symptomatic women, although 22% of the cases were diagnosed incidentally, either before surgery or through familial analysis.

Conclusion

This study examined HMB and obstetric complications in women with CFDs, analyzing data from 70 pregnancies as part of the PRO-RBDD study. The HMB occurrence did not differ between hypofibrinogenemia and dysfibrinogenemia. The rate of miscarriage and PPH in CFDs was substantially higher than that observed in the European and North American population. Although live birth rates are high in hypofibrinogenemia and dysfibrinogenemia, pregnancies can still be complicated by obstetric challenges, even in asymptomatic cases. In this study, no significant difference between obstetric complications was found between dysfibrinogenemia and hypofibrinogenemia. Early CFDs diagnosis before pregnancy, combined with the appropriate prescription of procoagulant and/or anticoagulant prophylaxis, may serve as an effective preventive strategy.

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Thesis conclusions

Rare bleeding disorders (RBDs) are inherited coagulation factor deficiencies that account for approximately 3–5% of all bleeding disorders. Due to their rarity, genotype–phenotype analyses have been performed in only a limited number of cases, and our current understanding of their genetic basis remains limited.

We investigated the mutational spectrum of RBDs using one of the largest cohorts collected over a 19-year period. This extensive dataset enabled the characterization of variants in 11 genes known to cause RBDs. Across all deficiencies—except afibrinogenemia and combined FV and FVIII deficiency—missense variants were the most common, and the majority affected the catalytic domain. By examining cases with reduced factor activity levels in each disorder, we aimed to explore genotype–phenotype correlations. In our cohort, no causative variant was identified in 4% of patients, and in nearly 8% of those with severe deficiency, a second expected pathogenic variant remained undetected. These findings highlight the need for further investigation using new tools such as long read sequencing to elucidate the underlying genetic mechanisms.

Congenital fibrinogen disorders (CFDs), accounting for about 8% of RBDs, are classified according to fibrinogen activity and antigen levels into quantitative (afibrinogenemia, hypofibrinogenemia) and qualitative (dysfibrinogenemia, hypodysfibrinogenemia) defects. Clinical history assessment of CFDs is essential before proceeding to specialized laboratory testing. Nevertheless, assessments of CFD patients using the ISTH-BAT and EN-RBD-BSS have been limited, and the results remain inconclusive. In this study, we applied both tools to a large CFD cohort. We found a moderate positive correlation between the two systems, and our results suggest that EN-RBD-BSS, alongside ISTH-BAT, is a sensitive tool for identifying CFD patients. Furthermore, when bleeding severity was classified according to previously established fibrinogen activity thresholds (asymptomatic: >100 mg/dL; Grade III: undetectable), severity grades were correctly identified in nearly two-thirds of patients.

Although CFDs are rare, the number of reported cases is growing. Integrating laboratory phenotype, genetic, and clinical data providing valuable insights into disease mechanisms and guiding treatment decisions. International collaborations and registries are crucial to improving patient care. We conducted a cross-sectional analysis within the Prospective Rare Bleeding Disorders Database (PRO-RBDD) to describe the laboratory, clinical, and genetic characteristics of a well-defined group of

CFD patients. The database includes demographic, clinical data and treatment from birth to enrollment, offering a comprehensive medical history for each individual. Using data from this large multicenter registry, this study confirmed that in addition to the ISTH-BAT, the EN-RBD-BSS is useful to identify CFD patients with a good level of sensitivity. Our results demonstrated that baseline factor activity accurately reflected bleeding severity in nearly two-thirds of patients.

Women and girls with CFDs face additional bleeding risk due to menstruation, pregnancy, childbirth, and the postpartum. However, due to the rarity of the disease, gynecologic and obstetric outcome data remain limited. Understanding the effects of CFDs on pregnancy, miscarriage, and postpartum hemorrhage (PPH) is vital for optimizing care and prevention strategies. In our analysis of 70 pregnancies in women with CFDs from the PRO-RBDD, the prevalence of heavy menstrual bleeding (HMB) did not differ between hypofibrinogenemia and dysfibrinogenemia. Miscarriage and PPH rates were substantially higher than those reported in European and North American populations. While live birth rates were high in both hypofibrinogenemia and dysfibrinogenemia, pregnancies were frequently complicated by obstetric events, even in asymptomatic patients. No significant differences in obstetric complications were observed between afibrinogenemia and hypofibrinogenemia. Early diagnosis before pregnancy, combined with tailored procoagulant and/or anticoagulant prophylaxis, may reduce these risks.

Overall, this work provides a comprehensive analysis of the genetic, laboratory, and clinical features of rare bleeding disorders, validates bleeding assessment tools for patient identification and bleeding severity grades, and highlights the significant gynecologic and obstetric risks in women with congenital fibrinogen disorders, emphasizing the importance of early diagnosis and tailored management.

Future advances in the diagnosis and treatment of RBDs will be driven by the integration of advanced genomic, laboratory, and therapeutic strategies. Long-read and whole-genome sequencing can identify variants missed by traditional methods, enabling more accurate diagnoses and earlier identification of at-risk individuals. Combining genetic data with laboratory phenotyping and clinical history allows personalized bleeding risk assessment, while next-generation coagulation assays can guide tailored therapy. Beyond traditional factor replacement, novel therapies, including engineered coagulation factors, gene- or RNA-based treatments, and emerging non-replacement approaches that enhance hemostasis, may provide safer, longer-lasting, and more convenient options, particularly for prophylaxis in high-risk situations. Together with international registries and real-world data, these

approaches promise more precise diagnosis, better risk prediction, and highly individualized treatment, ultimately improving patient outcomes and quality of life.

Riassunto

I disturbi emorragici rari (RBD) rappresentano circa il 3–5% di tutte le condizioni emorragiche ereditarie e comprendono carenze di fibrinogeno, protrombina e fattori FV, FVII, X, XI e XIII, per lo più ad ereditarietà autosomica recessiva. Questi disturbi derivano da varianti patogeniche nei geni dei singoli fattori. Tra i RBD, i disturbi congeniti del fibrinogeno (CFD) rappresentano circa l'8% e si distinguono in quantitativi (afibrinogenemia, ipofibrinogenemia) e qualitativi (disfibrinogenemia, ipodisfibrinogenemia). Questa tesi integra analisi genetiche, valutazioni cliniche standardizzate e fenotipizzazione di laboratorio, contribuendo a un significativo avanzamento nel campo.

(I) Nel primo capitolo abbiamo analizzato genotipo e fenotipo di laboratorio di 526 casi di RBD, una delle coorti più grandi fino a oggi studiata. La carenza di FVII è risultata essere la più comune (23%), mentre FII e FV+FVIII le più rare (6%). Tra 257 varianti geniche uniche, l'86% era patogena e l'11% mai riportata in precedenza; le varianti missenso predominano (57%), soprattutto negli esoni dei domini catalitici (48%). (II) Nel secondo capitolo abbiamo confrontato due sistemi di punteggio emorragico (ISTH-BAT e EN-RBD-BSS) per identificare pazienti con CFD e valutato la correlazione con livelli di fibrinogeno e gradi di severità. Entrambi gli strumenti hanno mostrato buona sensibilità, classificando correttamente la gravità clinica in quasi due terzi dei casi. (III) Nel terzo capitolo 166 casi di CFD sono stati caratterizzati tramite il Prospective Rare Bleeding Disorders Database, fornendo informazioni sui profili clinici, di laboratorio e genetici. I gradi di gravità emorragica corrispondevano alle soglie di attività dei fattori in quasi metà dei casi con difetti quantitativi di fibrinogeno. (IV) Nel quarto capitolo abbiamo esaminato le complicanze ginecologiche e ostetriche nelle donne con CFD. La coorte includeva 59 donne, con 70 gravidanze in 32 individui. L'ipermenorrea era il sintomo più frequente nell'afibrinogenemia (75% dei casi), ma anche emorragia post-partum (36%) e aborto spontaneo (23%) erano presenti in un elevato numero di casi. Non sono state osservate differenze significative nel rischio ostetrico tra donne disfibrinogeniche con o senza varianti geniche hotspot. Questi risultati sottolineano l'importanza di una diagnosi precoce e di eventuale profilassi, anche nei casi asintomatici.

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