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Novel *F8* and *F9* gene variants from the PedNet hemophilia registry classified according to ACMG/AMP guidelines

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Abstract

In hemophilia A and B, analysis of the *F8* and *F9* gene variants enables carrier and prenatal diagnosis and prediction of risk for the development of inhibitors. The PedNet Registry collects clinical, genetic, and phenotypic data prospectively on more than 2000 children with hemophilia. The genetic reports of *F8/F9* gene variants were classified uniformly to Human Genome Variation Society nomenclature and reevaluated using international population- and disease-specific databases, literature survey and, where applicable, computational predictive programs. We report 88 novel variants in the *F8* and *F9* genes, 80 fulfilling criteria for Class 5 (pathogenic), six for Class 4 (likely pathogenic) and two fulfilling criteria for Class 3 (variant of unknown significance) of the American College of Medical Genetics and Genomics/Association for Molecular Pathologyguidelines together with information on the respective phenotype and inhibitor formation. The study highlights the need to reevaluate and update earlier genetic reports in hemophilia both locally but also in variant databases in light of changed nomenclature and new guidelines.

KEYWORDS

F8 gene, *F9* gene, Factor IX, Factor VIII, hemophilia, variant database

1 | INTRODUCTION

Hemophilia A and B are X-linked recessive congenital bleeding disorders caused by pathogenic variants in, respectively, the *F8* or *F9* gene. Hemophilia A, caused by lack or dysfunction of the plasma protein Factor VIII (FVIII), affects about 1/5000 males, while hemophilia B, caused by lack or dysfunction of Factor IX (FIX), affects approximately 1/30,000 males (Mannucci & Tuddenham, 2001). Depending on the residual clotting activity in plasma levels of FVIII or FIX, hemophilia is categorized as severe (<1%), moderate (1–5%), or mild (6–40%). The cornerstone of hemophilia treatment is replacement therapy with FVIII/FIX concentrates and—recommended by the World Health Organization—treatment with prophylaxis in severe hemophilia (Andersson et al., 2017; Manco-Johnson et al., 2007). The main complication of replacement therapy is the development of anti-FVIII/FIX antibodies (inhibitors), which are able to neutralize the clotting activity of therapeutic clotting factors (Gouw et al., 2013).

Since the *F8/F9* variant type is the main determinant of plasma levels of FVIII or FIX, respectively, and disease severity, the analysis of the *F8* or *F9* gene variant in hemophilia patients and their families has become standard in hemophilia treatment centers in recent years. Knowledge of the variant allows genetic counseling and provides information on the risk of inhibitor development. In addition, information on clotting assays discrepancies, and in mild hemophilia A, the probability of a therapeutic response to DDAVP, can be retrieved (Goodeve & Peake, 2003; Seary et al., 2012). Sporadic cases, that is, with no known family history of hemophilia, account for approximately 30% of all cases when combining anamnestic data and haplotyping to reveal variants identical to descent (Halldén et al., 2012; Ljung et al., 1990). If hemophilia is diagnosed for the first time in a patient, studies show that new variants are found in around 70–80% of the mothers of these index cases (Ljung et al., 1991; Martensson et al., 2016).

Currently, direct gene sequencing either through Sanger or next-generation sequencing (NGS) methodologies is the predominant technique for the testing of single nucleotide variants and small insertions and deletions (Gomez & Chitlur, 2013). Nowadays, copy number variant analysis for large deletions and duplications is performed by NGS or complementary technologies such as array comparative genomic hybridization and multiplex ligation-dependent probe amplification. For the *F8* intron 22 inversion, Southern blot, long-range PCR, and inverse PCR protocols are used, while for the *F8* intron 1 inversion, a PCR-based method is the standard technique. The most common variant causing severe hemophilia A is intron 22 inversion in *F8* affecting approximately 40% of the patients but today a broad spectrum of more than 2000 variants causing hemophilia A and more than 1000 variants causing hemophilia B are described in FVIII or FIX variant databases, such as the American CDC Hemophilia Mutation Project databases CHAMP/CHBMP (https://www.cdc.gov/ncbddd/hemophilia/champs.html) or the European EAHAD Coagulation Factor Variant Databases (http://dbs.eahad.org), to which *F8 and F9* gene variants from all over the world are reported voluntarily by laboratories and clinicians (Li et al., 2013; Payne et al., 2013). The variant types in hemophilia cover a broad spectrum: in addition to the *F8* gene-specific inversion 22 and inversion 1, substitutions, deletions, duplications, and complex variants are found causing missense, nonsense, frameshift, deletion/insertion/duplication in frame, splice site variants and promotor variants. Usually, new variants are crosschecked with the above-named hemophilia variant databases, such as the European Coagulation Factor Variant Databases from EAHAD, the CDC-based CHAMP/CHBMP or Human Gene Mutation database (HGMD), which collect a large number of published gene alterations. In these databases, additional information about the number of patients with each reported variant and clinical information on severity of the disease, factor levels, and inhibitor development on every reported patient may be available (Li et al., 2013; McVey et al., 2020; Payne et al., 2013).

The clinical interpretation of a new or an unpublished genetic variant in the *F8* or *F9* genes, as well as other genes, should be based on guidelines published by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015). International *F8/F9* gene variant databases assist effective variant classification, especially when it is possible to combine such data with phenotypic and pedigree information. Various in silico prediction programs developed for missense or splice site variants may be helpful but the provided information should be interpreted with caution. When predicting the pathogenicity of a gene variant it is recommended to combine several prediction programs (Richards et al., 2015). Recently, a guideline specific for genetic analysis in bleeding disorders has been published (Gomez et al., 2019). In addition to hemophilia variant databases, the presence of a variant in a reference sequencing database of normal individuals is important and can be evidence of nonpathogenicity. These resources include the 1000 genomes, gnomAD, dbSNP, and the NHLBI exome sequencing project.

The PedNet Registry contains prospective data on children less than 18 years with hemophilia A or B born since January 1, 2000 who are followed up regularly in 31 hemophilia centers in 18 countries in Europe, Canada, and Israel. More than 2100 patients were included by 2019 and the *F8/F9* gene variant reported in 85% of the cases (Fischer et al., 2014). The purpose of the Registry is to promote and facilitate research and development of care in this large unselected patient population. The aim of this paper is to report all new *F8/F9* variants with clinical information on severity, factor level and inhibitor formation found in the PedNet Registry not previously published or known in hemophilia variant databases after reevaluation of the reported variant using the ACMG criteria of pathogenicity.

2 | METHODS

2.1 | The PedNet cohort

Data were retrieved from the "PedNet Registry," a database which is owned and administered by the "PedNet Haemophilia Research Foundation," consisting of 31 international hemophilia treatment

centers in 18 countries and registered at ClinicalTrials.gov at NCT02979119. A complete list of PedNet members is added in the Appendix. Approval for data collection was obtained from each center's ethical review board, and written informed consent was obtained from the parents or guardians of all participants, in accordance with the Declaration of Helsinki.

2.2 | Subjects

All patients with either hemophilia A or B, registered in the PedNet Registry by January 1, 2018 ($n = 1967$) were included. Data on patients' demographics, type and severity of hemophilia, and family history of hemophilia were collected. Reports on genotyping from the respective local genetic laboratories were collected from each single center.

2.3 | Nomenclature

All reports were then classified uniformly by a central genetic laboratory according to the recommendations of the Human Genome Variation Society (HGVS). The local laboratories predominantly used conventional Sanger sequencing of *F8* and *F9* genes and conventional analysis for inversions 22 and inversion 1 in the *F8* gene. Variant nomenclature was based on the following NCBI RefSec accession numbers and confirmed by Alamut and VariantValidator: *F8*: NM_000132.3; NG_011403.1; NP_000123.1 and *F9*: NM_000133.3; NG_007994.1; NP_000124.1 and GRCh37 genome build. All variants were cross-checked with the CDC-based databases CHAMP and CHBMP, the EAHAD *F8/F9* databases and the HGMD and a literature search on June 1, 2020, and only variants not described in these databases or published in a scientific journal searchable on Medline were included in this manuscript, referred to as "novel variants." Therefore, known polymorphisms or synonymous variants reported in patients with novel variants are not included in this analysis.

2.4 | Phenotype of hemophilia

The PedNet Registry follows the international classification for hemophilia valid when the Registry was initiated (i.e., severe form FVIII/FIX <1%, moderate 1–5%, and mild with 6–25%) and not the present classification where the mild form is defined as 6–40% (Blanchette et al., 2014). FVIII/FIX levels were measured at each participating center according to local standards. Both chromogenic and one-stage assay methods were accepted.

2.5 | Inhibitors

All patients in the PedNet Registry were included with baseline information at birth and their data were updated annually with regard to inhibitor status and exposure days up to the age of 18 years. Children included in this study were born between 2000 and 2017. Of the 97 patients included, 92 attained more than 50 exposure days to FVIII/IX concentrates. Inhibitors were divided into low-and high-titer inhibitors, defined as ≤5 Bethesda Units (BU) and more than 5 BU, respectively, according to international guidelines (Blanchette et al., 2014). In this study, inhibitors were reported for the new variants to support clinical information on this specific variant.

2.6 | Classification of reports on genotypes

In line with the established databases of CHAMP, CHBMP, and EAHAD, we used the following classifications:

The variant type in *F8* was classified as inversion 22, inversion 1, substitution, deletion, duplication, insertion, polymorphism, or complex variant.

The variant type in *F9* was classified as substitution, deletion, duplication, insertion, polymorphism, or complex variant.

The molecular consequence was reported in both *F8* and *F9* as missense, nonsense, frameshift, large deletion/insertion/duplication (>50 base pairs), small deletion/insertion/duplication (<50 base pairs), stop gain, in frame, silent variant, splice site variant, and promotor variant.

2.7 | In silico analyses

The deleterious effects of missense variants were assessed with ALAMUT VISUAL (http://www.interactive-biosoftware.com/alamut-visual/), a web-based tool, which allows simultaneous analysis by POLYPHEN-2 (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.bii.a-star.edu.sg), MutationTaster (http://www.mutationtaster.org), and Align GVGD (http://agvgd.hci.utah.edu/agvgd_input.php) and links to the databases ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and gnomAD (https://gnomad.broadinstitute.org/).

Variants at splice junctions were evaluated with ALAMUT VISUAL v.2.8.1 (http://www.interactive-biosoftware.com/alamut-visual/), which allows a simultaneous analysis with the programs SPLICE SITE FINDER-LIKE, MAXENT SCAN, NEURAL NETWORK SPLICE SITE, GENESPLICER, and HUMAN SPLICING FINDER. These tools were used together in accordance with guidelines for using prediction methods (Niroula & Vihinen, 2016). Missense variants close to splice sites underwent splice site prediction, too. If three or more of four prediction programs predicted that the variant under consideration was deleterious, it was accepted as a supporting criterion PP3 according to the ACMG guidelines. If three or more prediction programs predicted a benign variant, BP6 was used as the criterion. For splice site variants, four out of five prediction programs had to be significant to be accepted as a PP3 or a BP6 criterion, respectively.

2.8 | Classifying of new variants using the ACMG's criteria of pathogenicity

All new variants were classified using the published criteria of pathogenicity of the ACMG. For null variants the criterion PVS1 was used. The criterion PS3 was used for all patients, since well-established functional studies on FVIII or IX were available in all included variants, which are both validated, reproducible and robust in a clinical diagnostic laboratory setting and are specific for hemophilia. All reported new variants fulfilled criterion PM2 since no allele frequency was reported in gnomAD v2.1.1 (Karczewski et al., 2020). Criterion PM5 was used if a missense change at an amino acid residue where a different missense change determined to be pathogenic had been seen before. Criterion PP4 was used for all included variants since hemophilia has a single genetic etiology and a clear patient phenotype.

3 | RESULTS

Overall, 1967 patients from the PedNet Registry were included in the study. Of these, 1681 patients had a report on genotyping in the Registry (85.5%). Out of 1681 patients with hemophilia A or B, with all severities, 97 patients had 88 novel variants, of which 86 were classified as pathogenic or likely pathogenic: 69 causing hemophilia A and 17 hemophilia B; one variant in *F8* and one variant in *F9* gene were classified as variants of unknown significance; no benign or likely benign variants were found in the reported variants. Of the 86 likely disease-causing variants, 78 represent new unique variants present in only one patient. Eight variants were present in ≥2 patients; all of which were found in patients who were related family members with the same severity of hemophilia (e.g., brother and cousin). As expected, the majority of the new variants found were located in exon 14 in the *F8* gene and in exon 8 in the *F9* gene.

3.1 | F8—hemophilia A

In hemophilia A ($n = 70$), 39 of the new variants were substitutions, 25 were deletions, two were complex variants, three were duplications and one was an insertion (see Table 1). In mild and moderate hemophilia A, all new variants were "missense." Table 1 provides more detailed information on variant type and molecular consequence in the whole hemophilia A cohort.

Table 2 shows all null variants ($n = 39$) including nonsense variants, complex variants, duplications, insertions, and deletions found in patients with severe hemophilia. A total of 37 variants fulfilled Class 5 (pathogenic) in the ACMG classification; two variants—one deletion with small structural change in-frame and a duplication were classified as Class 4 (likely pathogenic).

Substitutions resulting in missense (Table 3) or at splice sites (Table 4) underwent in silico analysis with prediction programs, as described above. A total of 20 substitutions were classified as pathogenic (Class 5) and three as likely pathogenic (Class 4). Of eight splice site variants, seven were classified as pathogenic (Class 5) and one as a variant of unknown significance with contradicting criteria (PS3, PM2, PP4, and BP4). Inhibitors were diagnosed in 18/70 patients with hemophilia A with novel variants, all found in patients with the severe form of the disease, with the exception of p.Glu409Lys found in two related patients with moderate hemophilia, both of whom developed inhibitors.

TABLE 1 Type and molecular consequence of novel variants and phenotypic severity in hemophilia A

Variant type	Molecular consequence	All novel variants	ACMG Classes 4 and 5	Severe hemophilia A (<1% FVIII)	Moderate hemophilia A (1–5% FVIII)	Mild hemophilia A (5–25% FVIII)
Substitution	Missense	23	23	12	3	8
	Nonsense	11	11	11	0	0
	Splice site	5	5	5	0	0
Deletion	Large structure change (>50 bp)	1	1	1	0	0
	Small structure change (<50 bp, in frame)	1	1	1	0	0
	Frameshift	19	19	19	0	0
	Splice site	3	2	2	0	0
	Stop gain	1	1	1	0	0
Duplication	Frameshift	2	2	2	0	0
	Large structural change (>50 bp)	1	1	1	0	0
Insertion	Frameshift	1	1	1	0	0
Complex	Frameshift	2	2	2	0	0
Total no. variants		70	69	58	3	8

Abbreviations: ACMG, American College of Medical Genetics and Genomics; FVIII, Factor VIII.

TABLE 2 Null variants including nonsense variants, complex variants, duplications, insertions and deletions with frameshift or large/small structural change effect in hemophilia A and classification according to ACMG criteria

Exon	HGVS cDNA NM_000132.3 NG_011403.1	HGVS predicted protein changes NP_000123.1	Domain/chain	Allele frequency gnomAD	Inhibitor	Classifying criteria of pathogenicity (ACMG)	Clinical significance
4	c.437_438del	p.(Lys146Serfs*23)	A1/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
5	c.613A>T	p.(Lys205*)	A1/heavy chain	n.r.	No	PVS1, PS3 PM2, PP4	Pathogenic (Class 5)
6	c.738del	p.(Trp247Glyfs*11)	A1/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
7	c.812C>G	p.(Ser271*)	A1/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
7	c.871del	p.(Glu291Lysfs*7)	A1/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
7	c.952del	p.(Leu318Serfs*2)	A1/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
8	c.1025_1026del	p.(Tyr342Cysfs*17)	A1/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
8	c.1251dup	p.(Val418Ser*6)	A2/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
9	c.1412T>A	p.(Leu471*)	A2/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
10	c.1536del	p.(Gly513Valfs*2)	A2/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
11	c.1627del	p.(Ser543Glnfs*6)	A2/heavy chain	n.r.	Yes, HR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
13	**c.1965C>A**	**p.(Tyr655*)**	A2/heavy chain	n.r.	Yes, HR; no inhibitor	PVS1, PS3 PM2, PP4	Pathogenic (Class 5)
13	c.1978_1979insTTGT	p.(Ser660Ilefs*8)	A2/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.2859delinsGG	p.(Pro954Alafs*6)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.2897T>A	p.(Leu966*)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.3242del	p.(Asn1081Ilefs*57)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.3406del	p.(Ser1136Leu*2)	B/heavy chain	n.r.	Yes, HR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)

TABLE 2 (Continued)

Exon	HGVS cDNA NM_000132.3 NG_011403.1	HGVS predicted protein changes NP_000123.1	Domain/chain	Allele frequency gnomAD	Inhibitor	Classifying criteria of pathogenicity (ACMG)	Clinical significance
14	c.3436_3437del	p.(Lys1146Alafs*19)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.3597del	p.(Asp1199Glufs*19)	B/heavy chain	n.r.	Yes, LR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.3791del	p.(Ala1264Valfs*10)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.4675del	p.(Arg1559Aspfs*8)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.4804C>T	p.(Gln1602*)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.4998del	p.(Gln1666Hisfs*4)	B/heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.5008_5012del	p.(Thr1670Tyrfs*11)	a3/Heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.5016delinsGG	p.(Thr1673Aspfs*10)	a3/Heavy chain	n.r.	Yes, HR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
14	c.5032C>T	p.(Gln1678*)	a3/Heavy chain	n.r.	Yes, LR	PVS1, PS3 PM2, PP4	Pathogenic (Class 5)
14	c.5035G>T	p.(Glu1679*)	B/heavy chain	n.r.	Yes, HR	PVS1, PS3 PM2, PP4	Pathogenic (Class 5)
14	c.5058_5059del	p.(Val1689*)	a3/Heavy chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
15	c.5283del	p.(Phe1762Leufs*10)	A3/light chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
17	c.5716A>T	p.(Lys1906*)	A3/light chain	n.r.	Yes, HR	PVS1, PS3 PM2, PP4	Pathogenic (Class 5)
19	**c.6007G>T**	**p.(Glu2003*)**	A3/light chain	n.r.	No	PVS1, PS3 PM2, PP4	Pathogenic (Class 5)
22	c.6325del	p.(Arg2109Valfs*34)	C1/light chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
22	**c.6328C>T**	**p.(Gln2110*)**	C1/light chain	n.r.	Yes, HR; yes, HR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
22	**c.6373_6377del**	**p.(Ser2125*)**	C1/light chain	n.r.	Yes, LR; Yes, LR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
22	c.6410dup	p.(Asn2137Lysfs*24)	C1/light chain	n.r.	Yes, HR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
24	c.6719_6721del	p.(Pro2240del)	C2/light chain	n.r.	No	PS3, PM4, PM2, PP4	Likely pathogenic (Class 4)
25	c.6794del	p.(Gln2265Argfs*3)	C2/light chain	n.r.	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
15-23	c.(5209+1_5210-1)_(6574+1_6575-1)del	Del exon 15–23[a]	Multiple domains	n.r.	Yes, HR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)

(Continues)

TABLE 2 (Continued)

Exon	HGVS cDNA NM_000132.3 NG_011403.1	HGVS predicted protein changes NP_000123.1	Domain/chain	Allele frequency gnomAD	Inhibitor	Classifying criteria of pathogenicity (ACMG)	Clinical significance
22-25	c.(6273+1_6274-1)_(6900+1_6901-1)dup	Dup exon 22–25[a]	Multiple domains	n.r.	No	PS3, PM2, PP4	Likely pathogenic (Class 4)

Note: All variants found in patients with severe disease (FVIII <1%). HR = high-risk responder, >5 BU; LR = low-risk responder, <5 BU; variants in bold show variants found in more than one patient; n.r. = no allele frequency reported in gnomAD regarding cDNA and protein.

Abbreviations: ACMG, American College of Medical Genetics and Genomics; cDNA, complementary DNA; FVIII, Factor VIII.

[a]Breaking points not characterized.

3.2 | Hemophilia B

In hemophilia B patients, in total 18 new variants were found and 17 calssified as pathogenic or likely pathogenic (Table 5): 12 in patients with severe, three with moderate, and two with mild hemophilia B.

Table 6 shows that all null variants ($n = 12$) including nonsense variants, duplications, insertions, and deletions were classified as Class 5 (pathogenic) and were found in patients with severe hemophilia B with the exception of a patient with the moderate form who had a duplication causing frameshift.

Table 7 shows the substitutions leading to missense; all underwent in silico analysis with prediction programs, as described above, before classification. Of seven missense variants, five were classified as pathogenic (Class 5), one as likely pathogenic (Class 4), and one variant as a variant of unknown significance (Class 3), reported in Table 7.

In total, 16 variants in hemophilia B fulfilled Class 5 (pathogenic) in the ACMG classification, one variant Class 4 (likely pathogenic), and one as a variant of unknown significance with contradicting criteria (PS3, PM2, PP4, and BP4). One patient with a nonsense variant had developed an inhibitor (Table 6).

4 | DISCUSSION

Variant analysis in hemophilia has become a standard procedure over the years, confirming suspected hemophilia, making carrier diagnosis possible, and enabling the identification of variants with increased risk for the development of inhibitors. In this study encompassing data from 1681 children included in the PedNet Registry with hemophilia A or B, we report 88 novel variants in the *F8* and *F9* genes not previously reported in the HGMD or CHAMP, CHBMP, and EAHAD hemophilia variant databases by June 1, 2020. The novel variants were, as expected, frequently found in exon 14 of the *F8* gene and exon 8 in the *F9* gene, since both are the largest exons in *F8* and *F9*, respectively. No "hotspot" was identified, and the novel variants were of all types following the general spectrum seen in hemophilia A and B. This is in line with a report from Johnsen et al. (2017) in which 3000 hemophilia patients were investigated with NGS and 285 new variants were found in all variant types and *F8* or *F9* loci.

Of 88 novel variants, 80 could be ACMG classified as pathogenic (Class 5), six as likely pathogenic (Class 4), and two as variants of unknown significance (Class 3). As hemophilia is an X-linked single-gene disease with a well-established measurable phenotype, a variant is often found fulfilling Class 4 or 5 criteria for pathogenicity. Also in former studies, probable disease-causing variants are identified in approximately 95% of hemophilia A cases and in almost all patients with hemophilia B (Swystun & James, 2017). However, most likely not all variants considered to be "polymorphisms" or "not disease causing" in the *F8* or *F9* genes were included in the genetic reports reviewed in this paper. Following guidelines, it is recommended to report only Classes 4 and 5 pathogenicities to the

TABLE 3 Novel variants with missense effect in hemophilia A, their phenotypic severity, information on inhibitor and classified according to ACMG criteria

Exon	HGVS cDNA NM_000132.3 NG_011403.1	HGVS predicted protein changes NP_000123.1	Domain/ chain	Allele frequency gnomAD	Deleterious prediction/number of prediction programs	AA change in same codon/same aa reported[a]	Severity, FVIII activity (%)	Inhibitor prevalence	Classifying criteria of pathogenicity (ACMG)	Clinical significance
1	c.74A>C	p.(Tyr25Cys)	A1/heavy chain	n.r.	4/4	1/1	Moderate (4)	No	PS1, PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
1	c.97T>C	p.(Trp33Arg)	A1/heavy chain	n.r.	4/4	1/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
2	c.218T>C	p.(Phe73Ser)	A1/heavy chain	n.r.	3/4	1/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
4	c.424A>G	p.(Lys142Glu)	A1/heavy chain	n.r.	3/4	1/0	Mild (6)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
4	c.440T>G	p.(Val147Gly)	A1/heavy chain	n.r.	4/4	2/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
4	c.471G>T	p.(Trp157Cys)	A1/heavy chain	n.r.	4/4	0/1	Severe (0)	No	PS1 PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
4	c.548T>G	p.(Leu183Arg)	A1/heavy chain	n.r.	2/4	0/0	Mild (12)	No	PS3, PM2, PP4	Likely pathogenic (Class 4)
4	c.562A>C	p.(Asn188His)	A1/heavy chain	n.r.	4/4	2/0	Mild (15)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
7	c.827T>C	p.(Val276Ala)	A1/heavy chain	n.r.	4/4	1/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
8	**c.1225G>A**	**p.(Glu409Lys)**	A2/heavy chain	n.r.	4/4	2/0	Moderate (1) Moderate (2)	Yes, HR (both)	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
11	c.1601T>C	p.(Val534Ala)	A2/heavy chain	n.r.	4/4	1/0	Mild (7)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
11	c.1654T>G	p.(Tyr552Asp)	A2/heavy chain	n.r.	4/4	2/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
13	c.1963T>A	p.(Tyr655Asn)	A2/heavy chain	n.r.	3/4	2/0	Mild (7)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
13	c.2057C>A	p.(Thr686Lys)	A2/heavy chain	n.r.	2/4	1/0	Severe (0)	Yes, LR	PS1, PS3, PM2, PP4	Likely Pathogenic (Class 4)
13	c.2060T>A	p.(Leu687His)	A2/heavy chain	n.r.	4/4	2/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)

(Continues)

TABLE 3 (Continued)

Exon	HGVS cDNA NM_000132.3 NG_011403.1	HGVS predicted protein changes NP_000123.1	Domain/ chain	Allele frequency gnomAD	Deleterious prediction/number of prediction programs	AA change in same codon/same aa reported[a]	Severity, FVIII activity (%)	Inhibitor prevalence	Classifying criteria of pathogenicity (ACMG)	Clinical significance
15	c.5246T>C	p.(Phe1749Ser)	A3/light chain	n.r.	4/4	1/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
15	**c.5326G>A**	**p.(Gly1776Arg)**	A3/light chain	n.r.	4/4	1/0	Severe (0) Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
16	c.5374G>C	p.(Val1792Leu)	A3/light chain	n.r.	4/4	1/0	Mild (12)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
18	**c.5924T>G**	**p.(Ile1975Ser)**	A3/light chain	n.r.	4/4	3/0	Moderate (2) Moderate (2) Moderate (2)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
21	c.6212G>A	p.(Arg2071Lys)	C1/light chain	n.r.	4/4	3/0	Mild (9)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
24	c.6714G>T	p.(Trp2238Cys)	C2/light chain	n.r.	4/4	1/0	Severe (0)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
25	c.6821T>C	p.(Met2274Thr)	C2/light chain	n.r.	4/4	2/0	Mild (20)	No	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
26	c.7013T>C	p.(Leu2338Pro)	C2/light chain	n.r.	4/4	0/0	Severe (0)	Yes, LR	PS3, PM2, PP3, PP4	Likely pathogenic (Class 4)

Note: n.r. = No allele frequency reported in gnomAD regarding cDNA and protein; HR = high-risk responder, >5 BU; LR = low-risk responder, <5 BU; variants in bold show variants found in more than one patient; prediction programs used by Alamut: GVGD, SIFT, MutationTaster, PolyPhen.

Abbreviations: ACMG, American College of Medical Genetics and Genomics; cDNA, complementary DNA; FVIII, Factor VIII.

[a]Number of other reported amino acid changes in the same codon and number of reported same amino acid but different nucleotide.

TABLE 4 Novel splice site variants in hemophilia A with prediction score, phenotype severity, inhibitor development, and classified according to ACMG criteria

Exon/ intron	HGVS cDNA NG_011403.1	Allele frequency gnomAD	SSF score	MaxEnt score	NNSplice score	GeneSplicer score	HSF score	Severity, FVIII activity	Inhibitor	Classifying criteria of pathogenicity (ACMG)	Clinical significance
ex2	c.144_159del	n.r.	86.74 ⇒ –	11.18 ⇒ –	0.99 ⇒ –	10.23 ⇒ –	90.62 ⇒ –	Severe, 0	No	PVS1, PS3, PM2, PP3, PP4	Pathogenic (Class 5)
intr5	c.671-18_671-7del	n.r.	90.55 ⇒ 89.37 (−1.3%)	11.76 ⇒ 5.98 (−49.2%)	0.99 ⇒ 0.76 (−23.8%)	11.13 ⇒ 3.60 (−67.6%)	92.80 ⇒ 89.05 (−4.0%)	Mild (13)	No	PS3, PM2, PP4, BP4	VUS (Class 3)
intr7	c.1010-1G>A	n.r.	84.76 ⇒ –	7.11 ⇒ –	0.46 ⇒ –	5.73 ⇒ –	87.89 ⇒ –	Severe, 0	No	PVS1, PS3, PM2, PP3, PP4	Pathogenic (Class 5)
intr12	c.1904-1G>T	n.r.	95.70 ⇒ –	11.19 ⇒ –	0.89 ⇒ –	7.41 ⇒ –	93.77 ⇒ –	Severe, 0	No	PVS1, PS1[a], PS3, PM2, PP3, PP4	Pathogenic (Class 5)
ex14 /intr13	c.2114-2_2121del	n.r.	90.49 ⇒ –	11.73 ⇒ –	1.00 ⇒ –	6.88 ⇒ –	92.69 ⇒ –	Severe, 0	No	PVS1, PS3, PM2, PP3, PP4	Pathogenic (Class 5)
intr16	c.5586+1G>A	n.r.	85.71 ⇒ –	8.69 ⇒ –	1.00 ⇒ –	3.76 ⇒ –	93.07 ⇒ –	Severe, 0	Yes, LR	PVS1, PS1[a], PS3, PM2, PP3, PP4	Pathogenic (Class 5)
intr16	c.5587-1G>T	n.r.	82.12 ⇒ –	12.41 ⇒ –	0.96 ⇒ –	11.40 ⇒ –	88.34 ⇒ –	Severe, 0	Yes, LR,	PVS1, PS1[a], PS3, PM2, PP3, PP4	Pathogenic (Class 5)
intr17	c.5816-1G>T	n.r.	84.90 ⇒ –	9.65 ⇒ –	0.93 ⇒ –	6.27 ⇒ –	88.98 ⇒ –	Severe, 0	Yes, LR	PVS1, PS3, PM2, PP3, PP4	Pathogenic (Class 5)

Note: HR = high-risk responder, >5 BU; LR = low-risk responder, <5 BU; SSF, MaxEnt, NNSplice, GeneSplicer and HSF score as predicted by Alamut visual with following thresholds and score range in brackets as recommended by the program: SSF ≥70 [0–100], MaxEnt ≥0 [0–12], NNSplice ≥0.4 [0–1], GeneSplicer ≥0 [0–24], HSF ≥65 [0–100]; n.r. = no allele frequency reported in gnomAD regarding cDNA and protein.

Abbreviations: ACMG, American College of Medical Genetics and Genomics; cDNA, complementary DNA; FVIII, Factor VIII; mRNA, messenger RNA.

[a]According to ACMG guidelines: PS1 may also be used at supporting if different intronic change with the same or more severe splicing effect with respect to the predicted impact on the mRNA/protein.

TABLE 5 Type and molecular consequence of novel variants and phenotypic severity in hemophilia B

Variant type	Molecular consequence	All	ACMG Classes 4 and 5	Severe hemophilia B (<1% FIX)	Moderate hemophilia B (1–5% FIX)	Mild hemophilia B (5–25% FIX)
Substitution	Missense	7	6	2	2	2
	Nonsense	3	3	3	0	0
Deletion	Frameshift	4	4	4	0	0
Duplication	Frameshift	2	2	1	1	0
	Small del/ins/dup (<50 bp, in frame)	1	1	1	0	0
Insertion	Frameshift	1	1	1	0	0
Total		18	17	12	3	2

Abbreviations: ACMG, American College of Medical Genetics and Genomics; FIX, Factor IX.

clinician, and variants of unknown significance only if no other cause for the disease was found (Wallis et al., 2013). Thus, it is likely that more than the reported variant was found in some patients in the local laboratory, but only those variants interpreted as being significant were reported to the PedNet Registry.

One of the interpretation criteria in the ACMG classification is computational analysis with in silico analysis (PS3/BP4). Two of the presented variants were classified as a VUS due to contradicting in silico prediction. It is a known phenomenon that in silico analysis—despite being combined of several algorithms—can be nonconclusive and should be seen as only one step in categorizing variants as described by the ACMG (Niroula & Vihinen, 2016). Differential diagnosis should also be considered, for example, von Willebrand disease variants causing low FVIII levels.

While the ACMG guidelines' interpretation of variants offers a very useful, well-defined set of criteria in international consensus, further interpretation of the criteria can be required. Several publications have discussed how to interpret different criteria, for example, the US Sequence Variant Interpretation working group (https://www.clinicalgenome.org/working-groups/sequence-variant-interpretation/) or the UK Association for Clinical Genomic Science (https://www.acgs.uk.com/news/acgs-best-practice-guidelines-for-variant-classification-2019/). For bleeding disorders, a UK guideline by Gomez et al. (2019) is available. However, the interpretation of variants remains complex, and in 2017, evaluations from the National External Quality Assessment Service (UK) showed that laboratories rated new variants in different ways, in some cases differing between Classes 2 and 5. In another study, concordance of variant interpretation was only 34% in nine laboratories using both in-house criteria and ACMG guidelines, but this figure was raised after detailed review and consensus discussions to 71% (Amendola et al., 2016). As hemophilia is an X-linked disease with well-defined phenotypes and genotypes, phenotype association can be less demanding, but disease-specific interpretations and consensus discussions may be required to improve the final classification of the ACMG criteria.

Since the data were retrieved from the PedNet hemophilia Registry with 31 centers reporting over the last two decades, reporting of variants may differ between different laboratories over time, which is one of the limitations in our study. To ensure as high-quality reporting as possible in the Pednet Registry, all reports were reevaluated retrospectively and updated with HGVS nomenclature and classification in 2018–2020 by a genetic laboratory technician and two MDs (Lund University, Malmö/Lund, Sweden). A regular update of genetic reports is planned for the PedNet Registry and all new reports to be included are reevaluated continuously. The EAHAD database recently presented their new database with new data, analysis tools and common database architecture with new interfaces and filters that conform to HGVS guidelines and variants are now reported in relation to reference sequences (RefSeq; McVey et al., 2020). In addition, the EAHAD database plans to update annually.

While hemophilia genetic variant databases are very useful, it should be noted that they have certain limitations. Reporting in hemophilia variant databases, such as EAHAD, CHAMP, and CHBMP, is voluntary and reports are submitted from a wide spectrum of clinicians and laboratories, which makes the investigation of hemophilia population-based frequencies difficult. Also, the update of these registries may differ. To be sure that a variant is novel, a literature search has to be performed additionally. Entries to the HGMD are based on published variants; however, not all new variants are published. The definition of variant type and effect or molecular consequence differs between databases and publications and adaptations are needed for comparisons. There is no requirement to classify variants by the ACMG guidelines and to use prediction programs of missense or splice site variants when reporting such a variant, even if most new reports follow these standards today. Also, the type and amount of phenotypic and clinical data captured in these resources varies. As discussed by Gomez et al. (2019), some databases allow multiple reports of the same variant while others only report a single, usually the first, occurrence. This raises the possibility of the same variant being classified inconsistently depending on the sources of evidence used. Most probably, some variants in the hemophilia databases reported are variants not causing

TABLE 6 Null variants in hemophilia B including duplications, insertions, and deletions with frameshift or large/small structural change effect and substitutions with nonsense effect and classified according to ACMG criteria

Exon	HGVS cDNA NM_000133.3 NG_007994.1	HGVS predicted protein changes NP_000124.1	Domain/chain	Allele frequency gnomAD	Severity; FIX activity (%)	Inhi-bitor	Classifying criteria of pathogenicity (ACMG)	Clinical significance
1	c.54C>A	p.(Cys18*)	Signal peptide	n.r.	Severe (0)	Yes, HR	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
2	c.136del	p.(Arg46Glyfs*58)	Pro-peptide	n.r.	Severe (0)	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
3	c.273T>A	p.(Tyr91*)	GLA/light chain	n.r.	Severe (0)	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
4	c.345T>A	p.(Tyr115*)	EGF1/light chain	n.r.	Severe (0)	No	PVS1, PS3, PM2, PP4	Pathogenic (class 5)
4	c.360del	p.(Phe121Leufs*10)	EGF1/light chain	n.r.	Severe (0)	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
5	c.454_455insC	p.(Lys152Thrfs*8)	EGF2/light chain	n.r.	Severe (0)	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
6	c.632del	p.(Leu211Trpfs*34)	ACT-peptide	n.r.	Severe (0)	No	PVS1, PM2, PP4	Pathogenic (Class 5)
7	c.766_768dup	p.(Ile256dup)	Serine protease/ heavy chain	n.r.	Severe (0)	No	PS3, PM4, PM2, PP4	Pathogenic (Class 5)
8	**c.1054dup**	**p.(Tyr352Leufs*22)**	Protease/eavy chain	n.r.	Severe (0)Severe (0)	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
8	c.1295del	p.(Gly432Valfs*6)	Protease/heavy chain	n.r.	Severe (0)	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)
8	c.994dup	p.(Thr332Asnfs*7)	Protease/heavy chain	n.r.	Moderate (1)	No	PVS1, PS3, PM2, PP4	Pathogenic (Class 5)

Note: HR = high-risk responder, >5 BU; LR = low-risk responder, <5 BU; variants in bold show variants found in more than one patient (related); n.r. = no allele frequency reported in gnomAD regarding cDNA and protein.

Abbreviations: ACMG, American College of Medical Genetics and Genomics; cDNA, complementary DNA; FIX, Factor IX.

TABLE 7 Novel variants with missense effect in hemophilia B, their phenotypic severity, information on inhibitor and classified according to ACMG criteria

Exon	HGVS cDNA NM_000133.3 NG_007994.1	HGVS predicted protein changes NP_000124.1	Domain/chain	Allele frequency GnomAD	Deleterious prediction/number of prediction programs	Number of aa change in same codon/same aa reported[a]	Severity, FIX activity (%)	Classifying criteria of pathogenicity (ACMG)	Clinical significance
2	c.200A>G	p.(Glu67Gly)	GLA/light chain	n.r.	4/4	2/0	Moderate (2)	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
4	c.285T>A	p.(Asp95Glu)	EGF1/light chain	n.r.	2/4	3/0	Mild (15)	PS3, PM2, PM5, PP4	Likely pathogenic (Class 4)
6	c.536G>C	p.(Gly179Ala)	Linker/light chain	n.r.	4/4	2/0	Severe (0)	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
7	c.790A>G	p.(Thr264Ala)	Protease/ heavy chain	n.r.	4/4	2/0	Mild (6)	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
8	c.1005C>G	p.(Cys335Trp)	Protease/ heavy chain	n.r.	4/4	5/0	Moderate (1)	PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
8	c.1182G>T	p.(Met394Ile)	Protease/ heavy chain	n.r.	3/4	4/1	Severe (0)	PS1, PS3, PM2, PM5, PP3, PP4	Pathogenic (Class 5)
8	c.1370A>G	p.(Lys457Arg)	Protease/ heavy chain	n.r.	0/4	0/0	Mild (22)	PS3, PM2, PP4, BP4	VUS (Class 3)

Note: No inhibitors in this group. n.r = no allele frequency reported in gnomAD regarding cDNA and protein; prediction programs used by Alamut: GVGD, SIFT, MutationTaster, PolyPhen

Abbreviations: ACMG, American College of Medical Genetics and Genomics; cDNA, complementary DNA; FIX, Factor IX; mRNA, messenger RNA.

[a]Number of reported amino acid changes same amino acid but different nucleotide.

hemophilia, which was also suggested by another group, finding 11 earlier reported variants unlikely to cause hemophilia (Johnsen et al., 2017).

In 19 variants, inhibitor development was reported: 18 variants in patients with hemophilia A (18/70; 25.7%), and one in a patient with severe hemophilia B (1/19; 5.3%). Although the new variants only represent a subgroup of our population-based registry, this follows the expected rate of inhibitor formation for patients with hemophilia A and B. (Gouw et al., 2012). The risk of inhibitor development associated with a certain variant is very useful in clinical decision making on the type of prophylaxis or therapy in the more severe forms but also in the milder forms of the diseases (Mahlangu et al., 2018).

In conclusion, we report 88 novel variants in the *F8* and *F9* genes of which 86 are concluded to cause hemophilia A or B, according to the ACMG classification. The strength of our study is the uniform collection of variants in a large well-defined cohort with regular re-evaluation of genetic reports and alignment to international guidelines. This study also demonstrates the value of reevaluating and updating earlier genetic reports in the light of changed nomenclature, new classification criteria, such as ACMG guidelines, new in silico prediction programs, new or updated population databases and disease-specific databases, such as EAHAD and CHAMP.

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DATA AVAILABILITY STATEMENT

The *F8/F9* genetic reports used in this study are from the PedNet Registry governed by the nonprofit PedNet Haemophilia Research Foundation. The data that support the findings of this study are available from the registry of the PedNet Haemophilia Research Foundation. Restrictions apply to the availability of these data, which were used under license for this study. Data are available from the authors with the permission of PedNet Registry Foundation (www.pednet.eu). All variants presented in this paper are submitted to the EAHAD database (http://www.dbs.eahad.org) and publicly available.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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