Received: 31 August 2022 | Revised: 11 November 2022 | Accepted: 17 December 2022

https://doi.org/10.1016/j.rpth.2023.100036

ORIGINAL ARTICLE

F8/F9 variants in the population-based PedNet Registry cohort compared with locus-specific genetic databases of the European Association for Haemophilia and Allied Disorders and the Centers for Disease Control and Prevention Hemophilia A or Hemophilia B Mutation Project

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Funding information
This study is supported by the PedNet Haemophilia Research Foundation. Unrestricted sponsorship for the PedNet Haemophilia Foundation is currently received from Bayer AG, Novo Nordisk Healthcare AG, Pfizer SRL, CSL Behring

Abstract

Background: Hemophilia A and B are caused by variants in the factor (F) VIII or FIX gene. Selective reporting may influence the distribution of variants reported in genetic databases.

Objectives: To compare the spectrum of *F8* and *F9* variants in an international population-based pediatric cohort (PedNet Registry) with the spectrum found in the European Association for Haemophilia and Allied Disorders (EAHAD) and the Centers for Disease Control and Prevention Hemophilia A or Hemophilia B Mutation Project (CHAMP/CHBMP) databases.

Methods: All patients registered in the PedNet Registry on January 1, 2021 were included in this study. As comparators, data from patients with severe hemophilia included in the CHAMP/CHBMP registry (US center data) and EAHAD were used.

Results: Genetic information was available for 1941 patients. Intron 22 inversion was present in 52% of patients with severe hemophilia A; frameshift (36%), missense (28%), and nonsense (20%) were the most frequent variants in patients with severe hemophilia A who were inversion-negative. The most frequent variants in severe hemophilia B were missense (48%). In nonsevere disease, most variants were missense variants (moderate hemophilia A: 91%; mild hemophilia A: 95%, moderate and mild hemophilia B: 86% each). Comparison with the databases demonstrated a higher proportion of missense variants associated with severe hemophilia B in EAHAD (68%) than in PedNet (48%) and CHBMP (46%).

Conclusion: The PedNet population-based cohort provides an alternative to the established databases, which collect data by selective reporting, as it is a well-maintained

GmbH, Swedish Orphan Biovitrum AB (SOBI), Takeda, Hoffmann La-Roche.

Handling Editor: Dr Johnny Mahlangu

database covering the full spectrum of pathogenic *F8* and *F9* variants, and indicates the number of patients affected by each particular variant.

KEYWORDS
factor VIII, factor IX, hemophilia A, hemophilia B, genetic databases, population

Essentials

- Selective reporting of *F8/F9* variants may influence the distribution of variants in databases.
- We compared the spectrum of variants in a population with databases at risk for reporting bias.
- We showed the full spectrum of variants in the PedNet cohort and differences with other databases.
- This full spectrum of variants could serve as a population-based reference for studies.

1 | INTRODUCTION

The discovery of the genes encoding human coagulation factors, factor (F) VIII and FIX in the 1980s [1,2] enabled the identification of disease-causing variants in patients with hemophilia A and hemophilia B, respectively. The identification of a disease-causing variant in the FVIII/FIX genes (*F8/F9*) is important for determining carriership in women and for prenatal diagnosis, and it helps to predict the severity of hemophilia in the child. In addition, the variant type has been shown to predispose to inhibitor development [3].

Advances in sequencing techniques have increased the identification of variants and have prompted the creation of databases to increase the knowledge on the genetic background of hemophilia. General or central mutation databases such as Online Mendelian Inheritance in Man (OMIM) [4] and the Human Gene Mutation Database (HGMD) [5] contain a list of variants in all genes. However, the curators of these central databases are not necessarily experts in all the relevant genes. Therefore, these databases lack important characteristics for full clinical interpretation of a specific disease.

On the other hand, databases on a gene-by-gene basis (ie, locus-specific databases [LSDB]) are run by researchers with scientific expertise in a particular gene or phenotype and are a crucial tool for both diagnostic and research laboratories. The first LSDBs for *F8* and *F9* were established in the early 1990s [6,7]. Subsequently, web-based LSDBs such as the Haemophilia A Mutation, Structure, and Test Site (HAMSTeRS) and Hemobase [8–10] have been created. However, lack of available time and funding can have an impact on the maintenance of these databases, and if not updated regularly, a database loses its relevance. Moreover, these databases are not always easily accessible.

Therefore, the Centers for Disease Control and Prevention (CDC) compiled a variant list in an easily accessible format (CDC Hemophilia A Mutation Project [CHAMP] or Hemophilia B Mutation Project [CHBMP] database) and this list is updated quarterly with recently published variants [11,12]. In addition, evolving from previously developed databases, the European Association for Haemophilia and Allied Disorders (EAHAD) established a project with the aim of gathering single gene variant databases involved in clinical bleeding disorders and providing a single web portal to LSDBs for the genes related to hemostasis [13]. To date, over 3000 unique pathogenic variants causing hemophilia A and >1200 unique variants causing hemophilia B have been listed in the EAHAD coagulation factor variant database and CHAMP/CHBMP databases. These databases contain information on the variants previously reported in other LSDBs and those newly published in the literature. The EAHAD coagulation factor variant databases also include variants directly submitted by laboratories. However, selective reporting may influence the distribution of variants reported in the CHAMP/CHBMP and EAHAD databases. Moreover, data in the CHAMP/CHBMP databases are presented as a list of unique variants and not as a list of patients whose disease is caused by a particular variant, whereas the EAHAD database allows the data to be visualized both ways. In addition, as part of the Hemophilia Inhibitor Research Study [14], the CDC tested >1400 patients with hemophilia A and >220 patients with hemophilia B and reported their variant results in a separate Excel file (CHAMP/CHBMP United States [US] files).

The PedNet (European Paediatric Network of Haemophilia Management) Registry is a population-based prospective registry including all children with hemophilia born from January 1, 2000 onwards, diagnosed, treated and followed up at one of the participating centers. Among all the variables collected, *F8/F9* variants are included.

In this study, we described the full spectrum of pathogenic *F8/F9* variants in the PedNet cohort and compared how this population-based spectrum conformed to the spectrum of variants found in the established reference databases of EAHAD and CHAMP/CHBMP [11–13]. Furthermore, we showed that the data from the PedNet cohort could serve as a reference for studies in previously untreated patients (PUPs).

2 | MATERIALS AND METHODS

2.1 | Study population and patient data collection

The PedNet Registry is based on the prospective collection of clinical, genetic, and phenotypic data of PUPs with hemophilia A or hemophilia B

born from January 1, 2000 onward and is registered on http://ClinicalTrials.gov under the number NCT02979119. The included patients were diagnosed in one of the 33 collaborating hemophilia treatment centers from 18 countries (Europe, Canada [2 centers] and Israel [1 center]). Ethical approval was obtained in each of the centers and written informed consent was obtained from the parents/caregivers before inclusion in the Registry in accordance with the Declaration of Helsinki.

2.2 | Hemophilia severity

FVIII/FIX levels were measured at least twice, locally at the participating center, by either chromogenic or one-stage assay methods and the severity of hemophilia was defined. The PedNet Registry follows the international classification for hemophilia for severe (FVIII/FIX <1%), and moderate hemophilia (FVIII/FIX, 1%–5%) [15]. For mild hemophilia, only patients with factor levels 6%–25% were included [16].

2.3 | Genotyping and classification

Genotyping was performed locally by each center's established routines, testing for inversions and predominantly using Sanger sequencing. In more recent years, next-generation sequencing has been used in some centers. All genetic reports provided to the coordinating center were checked and revised according to the recommendations of the Human Genome Variation Society. In addition, all genetic variants were classified using the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) criteria and terminology [17,18]. In most genetic reports, only the pathogenic or likely pathogenic variant was reported, but in some cases second variants, eg, polymorphisms, were reported. In this article, only the pathogenic variants causing hemophilia A or hemophilia B were used.

In line with the established databases of CHAMP/CHBMP and EAHAD, the following classifications are used in the PedNet database:

- The variant type in *F8* was classified as intron 22 inversion, intron 1 inversion, 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 (variant effect) was classified in both *F8* and *F9* as missense, nonsense, frameshift, large or small (> or <50 base pairs) deletion/insertion/duplication, silent variant, splice site variant, promoter variant, intron variant, polymorphism (missense/splice site/silent) and inversion.

2.4 | Data extraction from the PedNet database

For the current analysis, data extraction was performed on January 1, 2021 and included information on the type of hemophilia, severity, gender, family history, variant type, and molecular consequence. Patients without a known family history for hemophilia were considered sporadic cases. Patients with a variant effect other than intron 1 or intron 22 inversion were considered inversion-negative patients.

2.5 | Data extraction from the EAHAD database and CHAMP/CHBMP US files

For comparison, data from the international hemophilia databases EAHAD and CHAMP/CHBMP on severe hemophilia were collected. Both registries, EAHAD and CHAMP/CHBMP, were contacted before data extraction and gave permission for the use of the publicly available data. For the CHAMP/CHBMP registry, US center data were used because these are cohort-collected data—in contrast to the international database which includes data on each variant found. The extraction date for the EAHAD data was January 3, 2021.

3 | RESULTS

3.1 | PedNet study population and spectrum of variants

At the time of data extraction, 2278 boys and 19 females were included in the PedNet Registry. For this study, females were excluded from further analysis. Information on the *F8* or *F9* variant was available for 1941 patients (85%): 86% (1631/1904) patients with hemophilia A and 83% (310/374) patients with hemophilia B. With respect to disease severity, genetic analysis was performed in 92%, 77%, and 78% of patients with severe, moderate, and mild hemophilia A, and in 88%, 84%, and 76% of patients with severe, moderate, and mild hemophilia B, respectively. No disease-causing variants were found in 35 patients with hemophilia A and 5 patients with hemophilia B (2% and 1%, respectively). Genetic testing was performed in 84% of patients with hemophilia A with a known family history (n = 898/1073), in 89% of sporadic cases (n = 696/786), and in 82% of patients without the information on family history (n = 37/45). In addition, genetic testing was performed in 80% patients with hemophilia B having a known family history (n = 187/234), in 88% of sporadic cases (n = 116/132), and in 88% of patients without the information on family history (n = 7/8). The distribution of variant effects is shown in Table 1 (for hemophilia A) and Table 2 (for hemophilia B).

3.1.1 | Hemophilia A

A disease-causing variant was identified in 1151 of 1170 (98%) patients with severe hemophilia A. The most frequent variant type was an intron 22 inversion, found in 597 of the 1151 patients (52%). The intron 1 inversion occurred in 17 patients (1%). In noninversion patients (n = 537), variant effects included 194 frameshift (36%; mostly affecting exon 14), 151 missense (28%; mostly affecting exons 23 and

TABLE 1 Distribution of variant effects across severities in boys with hemophilia A, included in the PedNet Registry.

	Missense	Nonsense	Silent	Splice site	Promoter	Frameshift	Small structural change (<50 bp)	Large structural change (>50 bp)	Intron	Inversion	Total
Severe	151 (13)	110 (9.6)		38 (3.3)	1 (0.6)	194 (16.9)	3 (0.3)	40 (3.5)		614 (53.3)	1151
Moderate	157 (91.3)	1 (0.6)	1 (0.6)	6 (3.5)		5 (2.9)	1 (0.6)			1 (0.6)	172
Mild	293 (95.1)		1 (0.3)	3 (1.0)	3 (1.0)		2 (0.6)	4 (1.3)	2 (0.6)		308
Total	601	111	2	47	4	199	6	44	2	615	1631

Only male patients with a known variant are shown in the Table.

Data are presented as numbers (percentage). Detailed information on ethnicity is not available in the PedNet Registry.

26), 110 nonsense (21%; mostly affecting exon 14), and 38 splice site variants (7%), 40 large structural changes (7%), 3 small structural changes (0.6%; in exons 15, 22 and 24), and 1 promoter variant (0.2%). The causative variant remained unknown in 19 patients (2%).

In patients with both moderate (*n* = 172) and mild (*n* = 308) hemophilia A, missense variants encompassed the most common variant effect and accounted for 157 of the moderate (91%) and 293 of the mild cases (95%). However, although among patients with moderate hemophilia A these variants were almost equally distributed between the A1 (19%), A2 (23%), A3 (21%), and C1 (21%) domains, they were located most frequently in the A2 domain (40%) in patients with mild hemophilia A (Figure 1A).

3.1.2 | Hemophilia B

In patients with severe hemophilia B (*n* = 160), the most frequent variant type was a pathogenic point mutation (*n* = 123; 77%) and when stratified according to the variant effect, we found mostly missense (*n* = 77; 48%) and nonsense variants (*n* = 34; 21%). In addition, large structural changes (*n* = 18; 11%), frameshift (*n* = 14; 9%), promoter and splice site variants (*n* = 7; 4% each), and small structural changes (*n* = 3; 2%) were found. The disease-causing variant was not identified in one patient. The genetic variants were spread throughout *F9*.

In both moderate (*n* = 79) and mild (*n* = 71) hemophilia B, missense variants represented the causative variant effect in most patients: 68 of moderate and 61 of mild cases (86% each). As in hemophilia A, the distribution of these variants differed between moderate and mild hemophilia B. Most missense variants occurred in the serine protease domain in both groups (51% for moderate hemophilia B and 60% for mild hemophilia B). Yet, mild hemophilia B was associated more frequently with variants in the EGF1-domain whereas variants in the pro-peptide or linker were found less frequently (Figure 1B). The distribution of missense variants between the heavy and light chains was very similar among patients with hemophilia B.

In addition, 16/17 (94%) variants in the *F9* promoter were reported to be associated with a hemophilia B Leyden phenotype.

3.2 | Spectrum of variants in the PedNet cohort according to family history

We also compared the distribution of variant effects in the PedNet cohort between sporadic cases of severe hemophilia and those with a known family history. Missense variants were significantly less frequent in sporadic cases of severe HA (61/614 (10%) versus 87/515 (17%) in cases with a known family history; p=0.0007). In contrast, there were no significant differences in the distribution of variant effects in patients with severe hemophilia B, according to the family history.

3.3 | Spectrum of variants compared with EAHAD database and CHAMP/CHBMP files

In this part of our study, we restricted the analysis to patients with severe hemophilia.

TABLE 2 Distribution of variant effects across severities in boys with hemophilia B, included in the PedNet Registry.

	Missense	Nonsense	Silent	Splice site	Promoter	Frameshift	Small structural change (<50 bp)	Large structural change (>50 bp)	Polymorphism	Total
Severe	77 (48.1)	34 (21.3)		7 (4.4)	7 (4.4)	14 (8.8)	3 (1.9)	18 (11.3)		160
Moderate	68 (86.1)	1 (1.3)		3 (3.8)	4 (5.1)	2 (2.5)			1 (1.3)	79
Mild	61 (85.9)			3 (4.2)	6 (8.5)					70
Total	206	35		13	17	16	3	18	1	309

Only male patients with a known variant are shown in the Table.

Data are presented as numbers (percentage).

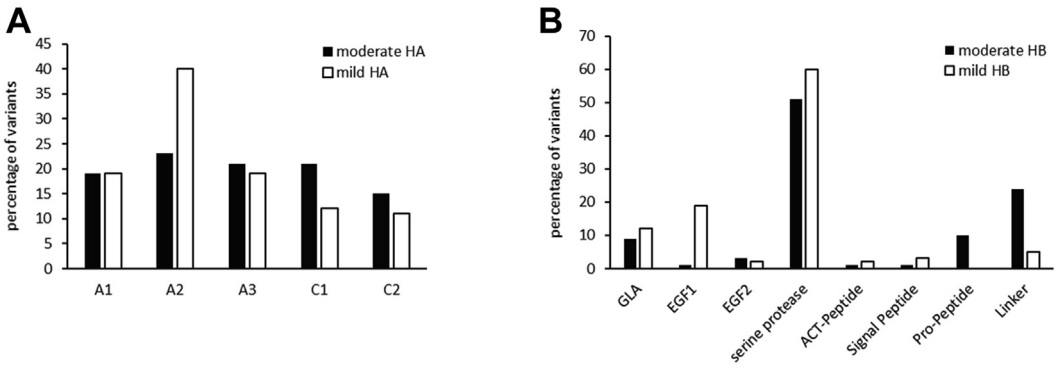

FIGURE 1 Location of missense variants in moderate and mild hemophilia. (A) Location according to the factor VIII protein domains in moderate or mild hemophilia A; (B) Location according to the factor IX protein structure in moderate or mild hemophilia B.
GLA: c-carboxy glutamic acid domain; EGF: epidermal growth factor like domain; ACT-Peptide: activating peptide

3.3.1 | Hemophilia A

The EAHAD database does not list *F8* inversions. It contained molecular information on 4691 severe, inversion-negative patients with hemophilia A. The most frequent variant effects in these patients were frameshift (*n* = 1487; 32%), missense (*n* = 1418; 30%), and nonsense variants (*n* = 966; 21%) followed by splice site variants and large structural changes.

The US cohort consisted of 668 patients with severe hemophilia A, of whom 288 (43%) had an intron 22 inversion, 11 (2%) had an intron 1 inversion; in 15 patients (2%) the causative variant was not found. In inversion-negative patients, the most predominant variant effects were frameshift (*n* = 108; 31%), missense (*n* = 100; 28%), and nonsense variants (*n* = 79; 22%), followed by large structural changes (*n* = 42; 12%).

For the comparison between the PedNet cohort and the EAHAD database and CHAMP/CHBMP US files, only inversion-negative patients with severe hemophilia A were included. The spectrum in inversion-negative patients with severe hemophilia A was almost identical between the databases (Figure 2).

3.3.2 | Hemophilia B

In 3823 patients with severe hemophilia B included in the EAHAD database, missense variants (*n* = 2609; 68%) were the most frequently identified variant effects; nonsense variants represented the causative variant effect in 592 (15%) patients with severe hemophilia B.

In the US cohort, missense variants (*n* = 40; 46%) accounted for almost half of the variant effects in 87 patients with severe hemophilia B, whereas nonsense variants occurred in 19 patients (22%).

Comparing the PedNet cohort to the EAHAD and CHBMP database, missense variants were the most prevalent variant effects in each database (Figure 2).

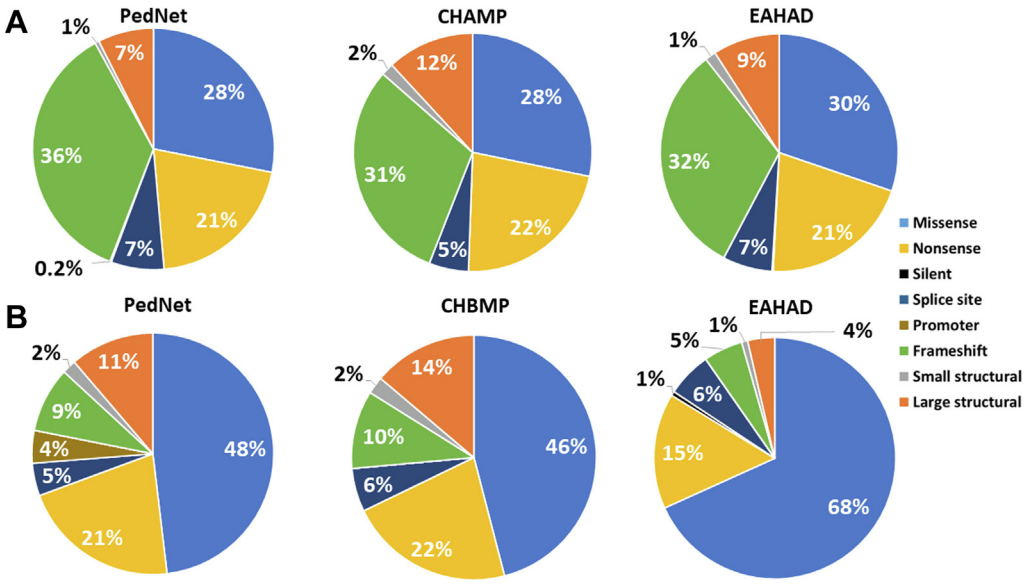

FIGURE 2 Spectrum of variant effects in the PedNet cohort compared with the CHAMP/CHBMP US files and EAHAD database. (A) inversion-negative patients with severe hemophilia A; (B) severe hemophilia B patients. Data are presented in percentages.

4 | DISCUSSION

We report on the *F8/F9* variants in a population-based cohort of 1941 patients affected with hemophilia A or hemophilia B, included in the PedNet registry and propose this database as an alternative to the established LSDBs.

First of all, we found that 50% of our patients with severe hemophilia A had an intron 22 inversion, compared with 43% in the CHAMP-US cohort and a widely accepted prevalence of approximately 45% [19]. However, the PedNet cohort is the first large and population-based cohort providing data on the prevalence of intron 22 inversions and should therefore be considered as an additional benchmark. Additionally, the distribution of variant effects in inversion-negative patients with severe hemophilia A was very similar between the PedNet cohort and the EAHAD database and CHAMP-US file: frameshift, missense, and nonsense variants were the most frequent variant effects in these patients. In patients with severe hemophilia B, missense variants occurred most commonly, a finding that was again comparable to the other databases.

Next, we studied the distribution of variants in patients with nonsevere hemophilia. Although missense variants were the most frequent variant effects both in patients with moderate and mild hemophilia, we found that their location differed depending on the severity of the disease. Indeed, missense variants associated with mild hemophilia A were located most frequently in the A2 domain, whereas these were more evenly distributed between the A1, A2, A3, and C1 domains in moderate cases. Consequently, the heavy chain was more likely to be affected in patients with mild hemophilia A than in those with moderate hemophilia A. For hemophilia B, variants in mild hemophilia B occurred more frequently in the EGF1 domain but less frequently in the pro-peptide and linker than in moderate hemophilia B. The impact of variants on the FVIII or FIX protein structure and consequently on the disease severity is a topic of current research [20–23] and evidence is emerging that phenotypic variation may also be related to the region where the variant occurs. [20,21] However, the role of the location of the variant warrants further exploration.

In addition, no disease-causing variant could be identified in the respective genes in 2% of our patients with hemophilia A and 1% of patients with hemophilia B, which is comparable with the CHAMP data and with some reports [24–26], but low in comparison with other studies, that failed to identify a disease-causing variant in up to 11% of the patients. [27–31] Of note, recent evolutions in genetic testing have improved variant detection and may explain the differences between various studies.

Furthermore, we also evaluated whether the spectrum of variants in severe hemophilia was different according to the family history. Interestingly, missense variants were less common in sporadic cases of severe hemophilia A, whereas in patients with severe hemophilia B no differences were observed. A study by Lu et al. [32] reported similar results in patients with hemophilia B, but a comparison of the spectrum between sporadic and familial cases has not yet been studied extensively. Note that the definition of sporadic cases in our cohort is based on the lack of other symptomatic/diagnosed cases in the family rather than on the genetic test result of the patient's mother. Importantly, haplotyping in hemophilia B has shown that, particularly in mild disease, seemingly sporadic cases could be related without anyone knowing [33], which may influence our observations.

Finally, in view of inhibitor development, it is extremely important to have a correct variant spectrum frame to interpret the results of studies in PUPs in the light of their representation as the hemophilia population. Three LSDBs are currently used: CHAMP (*F8* variants), CHBMP (*F9* variants), and EAHAD coagulation factor variant databases (*F8* and *F9* variants). The development of these databases has much improved the accessibility of information on the location of a variant within the gene, the associated disease severity, and the risk of inhibitor development. In addition, all variants described in these databases conform to a common nomenclature following the Human Genome Variation Society guidelines. Nevertheless, these LSDBs have some limitations. First, they have been built starting from previous LSDBs or central mutation databases, supplemented and updated with variants found through literature searches or through submission by individual laboratories or researchers. Therefore, a reporting bias can affect their accuracy and completeness. Next, the CHAMP/CHMBP Mutation Lists are listings of variants. As they do not indicate the number of patients whose disease is caused by a particular variant, extrapolating the frequency of a variant to frequencies of patients affected by that variant is not possible based on these data. Indeed, the CHAMP Mutation List only includes 2 listings for inversion (ie, intron 1 inversion and intron 22 inversion), although over 40% of cases of severe hemophilia A are caused by inversions. On the contrary, in the CHAMP/CHBMP US files, each row does correspond to a patient included in the population-based Haemophilia Inhibitor Research Study Investigators [14]. Finally, *F8* inversions are not listed in the EAHAD database. In this study, we report the full spectrum of *F8/F9* variants in a multicenter large population-based cohort of PUPs affected with hemophilia A or B. Therefore, the spectrum of variants presented here can serve as a population-based reference and could be useful in future studies, although the database itself is not freely accessible.

Our study has some limitations. First, the PedNet Registry only collects data from patients younger than 18 years. Additionally, patients with mild hemophilia and FVIII or FIX levels above 25% were not included. Thus, patients with mild hemophilia and/or a less severe phenotype may be underrepresented, especially if there was no known family history. However, in 2001 and again in 2014, the Scientific Subcommittee on Factor VIII and Factor IX and Rare Coagulation Disorders of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis recommended classifying hemophilia as mild if the plasma FVIII or FIX levels were between 5% and 40% [15,34]. Therefore, the EAHAD coagulation factor variant databases and the CHAMP/CHBMP US files may include patients with mild hemophilia having FVIII or FIX levels between 25% and 40%, whereas the PedNet registry does not. Second, some females with hemophilia are included in the PedNet Registry, but they most likely do not represent all female patients with hemophilia. Therefore, data on females have not been analyzed in this study. Additionally, detailed information on ethnicity is not available and although we

included patients from Europe, Canada and Israel, some ethnic groups may be underrepresented. In addition, we do not report on second variants. Although 2 pathogenic variants are probably rare and the clinical significance is still unresolved [35–38], they should be considered in patients with a known disease-causing variant but unusual phenotype, but also in genetic counseling settings. Finally, genetic analysis has not been performed in some patients. Patients with severe hemophilia A and severe or moderate hemophilia B were more likely to be tested but genetic testing was less frequently performed in patients with severe hemophilia A having a known family history, most likely because the disease-causing variant was already known. However, reporting the variant is not allowed in the PedNet Registry, unless confirmed in the patient himself. This may have caused a bias in our data.

In conclusion, the PedNet population-based cohort provides an alternative to the established databases, which collect data by selective reporting, as it is a well-maintained database covering the full spectrum of pathogenic *F8* and *F9* variants, and indicates the number of patients affected by each particular variant.

ACKNOWLEDGMENTS

All members of PedNet since the start of the PedNet Registry have been collaborators and contributed with basic data and regular follow-up data on their respective patients. The members' significant commitment has been of crucial importance and is greatly acknowledged. We specifically thank all current PedNet Study Group Members MT Alvarèz Román, Unidad de Coagulopatías, Hopital Universitario La Paz, Madrid, Spain; O Benítez-Hidalgo, Unitat Hemofilia, Hospital Vall d'Hebron, Barcelona, Spain; HM van den Berg, PedNet Haemophilia Research Foundation, Baarn, The Netherlands; J Blatny, Department of Paediatric Haematology, Children's University Hospital, Brno, Czech Republic; M Bührlen, Gesundheit Nord, Klinikum Bremen Mitte, Prof.-Hess-Kinderklinik, Bremen, Germany; M Carcao, Division of Haematology/Oncology, Hospital for Sick Children, Toronto, Canada; M Carvalho, Serviço de Imunoterapia, Centro de Referência de Coagulopatias Congénitas, Centro Hospitalar e Universitário São João, E.P.E., Porto, Portugal; E Chalmers, Department of Haematology, Royal Hospital for Sick Children, Yorkhill, Glasgow, UK; H Chambost, APHM, La Timone Children's Hospital, Center for Bleeding Disorders & Aix Marseille Univ, INSERM, INRA, C2VN, Marseille, France; A Rosa Cid, Unidad de Hemostasia y Trombosis, Hospital Universitario y Politécnico La Fe, Valencia, Spain; S Claeyssens, Centre Regional d'Hemophilie, Centre Hospitalo Universitaire, Toulouse, France; C Escuriola, HZRM Hämophilie Zentrum Rhein Main GmbH, Mörfelden-Walldorf, Germany; K Fischer, Van Creveld Kliniek, University Medical Center Utrecht, Utrecht, The Netherlands; C Van Geet, Catholic University of Leuven, Campus Gasthuisberg, Service of Pediatric Haematology, Leuven, Belgium; H Glosli, Oslo University Hospital HF, Oslo, Norway; C Königs, University Hospital Frankfurt, Department of Paediatrics and Adolescent Medicine, Frankfurt, Germany; M Koskenvuo, New Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland; K Kurnik, Dr. V. Hauner Children's Hospital, University of Munich, Munich, Germany; R Liesner, Hemophilia Center, Department of Haematology, Great Ormond Street Hospital for Children, London, UK; G Kenet, National Hemophilia Center Sheba Medical center, Tel Hashomer & Amalia Biron Research Institute of Thrombosis & Hemostasis, Tel Aviv University, Israel; C Male, Department of Paediatrics, Medical University Hospital of Vienna, Vienna, Austria; A Molinari, Dipartimento di Ematologia ed Oncologia, Unità Trombosi ed Emostasi, Ospedale Pediatrico Giannina Gaslini, Genova, Italy; J Motwani, Department of Haematology, The Children's Hospital, Birmingham, UK; B Nolan, Department of Paediatric Haematology, Our Lady's Children's Hospital for Sick Children, Crumlin, Dublin, Ireland; R d'Oiron, Centre de Référence pour le Traitement des Maladies Hémorragiques (CRTH), Hôpital Bicêtre, Kremlin Bicêtre AP-HP, France; J Oldenburg, Institut für Experimentelle Hämatologie und Transfusionsmedizin, Universitätsklinikum Bonn, Germany; H Pergantou, Haemophilia-Haemostasis Unit, St. Sophia Children's Hospital, Athens, Greece; S Ranta, Department of Pediatrics, Clinic of Coagulation Disorders, Karolinska Hospital, Stockholm, Sweden; J Rössler, Inselspital Bern, University Children's Hospital, Division of Paediatric Haematology, Bern, Switzerland; G Rivard, Division of Hematology/Oncology, Hôpital St Justine, Montréal, Canada.

We also thank the EAHAD and CHAMP/CHBMP databases for kind permission to use data for this publication. Finally, the authors greatly appreciate the support of the staff of the PedNet Haemophilia Research Foundation, especially Marloes de Kovel and Ella van Hardeveld.

FUNDING

This study is supported by the PedNet Haemophilia Research Foundation. Unrestricted sponsorship for the PedNet Haemophilia Foundation is currently received from Bayer AG, Novo Nordisk Healthcare AG, Pfizer SRL, CSL Behring GmbH, Swedish Orphan Biovitrum AB (SOBI), Takeda, Hoffmann La-Roche.

AUTHOR CONTRIBUTIONS

All authors have participated in the concept and design; analysis and interpretation of data; drafting and/or revising of the manuscript. Each author listed on the title page of the manuscript has approved the submission of this version of the manuscript and takes full responsibility for the manuscript.

RELATIONSHIP DISCLOSURE

V.L. has been as a speaker and/or advisor for Bayer, Novartis, NovoNordisk, Octapharma, Roche, Sobi, and Takeda; V. L. has received financial support for travel, accommodations, and expenses from Jazz Pharmaceuticals, Roche, and Sobi. M.E.M. has acted as paid consultant, speaker and/or advisor for Bayer, Biomarin, CSL Behring, Catalyst Biosciences, Grifols, Kedrion, LFB, Octapharma, Novo Nordisk, Pfizer, Roche, Sanofi, Sobi, Spark Therapeutics, Takeda, and UniQure. M. K-K. has played a consulting or advisory role for Bayer, Sobi, and Takeda; M. K-K. has received research funding from

NovoNordisk and financial support for travel, accommodations, and expenses from Bayer, NovoNordisk, Sobi, and Takeda. R. L. has received compensation for consultancy work (DMC, Advisory Board) or remuneration for lectures from NovoNordisk, Roche, Sanofi, Sobi, and Takeda. N. G.A. has served as a speaker and/or on advisory boards for Bayer, CSL Behring, Octapharma and Sobi. None of these conflicts of interest is relevant to this paper.

DATA AVAILABILITY

The data that support the findings of this study are available from 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 the PedNet Haemophilia Research Foundation.

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