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REVIEW

Genetic diagnosis in hemophilia and von Willebrand disease

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ABSTRACT

Phenotypic assays are first-line in terms of diagnostic testing for inherited bleeding disorders. However, since the characterization of the genes that encode coagulation factors in the 1980s, significant progress has been made in translating this knowledge for diagnostic and therapeutic purposes. For the hemophilias, molecular genetic testing can be used to determine carrier status, establish a prenatal diagnosis and predict the likelihood of inhibitor development or anaphylaxis in response to infused coagulation factor concentrates. In contrast, for von Willebrand disease (VWD), significant recent advances in our understanding of the molecular genetic basis of the disease have allowed for the development of rational approaches to genetic diagnostics. Questions remain however, about this complex genetic disorder and how to incorporate emerging knowledge into diagnostic strategies.

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1. Introduction

Collectively, inherited bleeding disorders may affect over 7.5 million individuals worldwide, with von Willebrand disease (VWD) being the most common overall, and the hemophilias being the most frequently diagnosed severe bleeding disorders [1–4]. Historically, diagnoses of these disorders were performed through biochemical analyses and functional mixing studies to identify patient-specific clotting factor deficiencies. The genes encoding human coagulation factors FVIII (F8), FIX (F9), and von Willebrand factor (VWF) were cloned in the early to mid-1980s, allowing for the identification of disease-causing variants in patients with quantitative coagulation factor deficiencies [5–11]. The development of PCR increased the speed and availability of molecular testing [12], and genetic diagnosis for inherited bleeding disorders emerged as a routinely utilized complementary diagnostic modality shortly thereafter. Today, patient genotyping for hemophilia and VWD assists with both the diagnosis and bleeding management of these diseases, making it an invaluable clinical tool [13].

2. The value of genetic testing for inherited bleeding disorders

In hemophilia, the identification of the underlying F8 or F9 mutation is important for carrier and prenatal diagnosis as well as prediction of risk for inhibitor formation or anaphylactic reaction following infused replacement therapy [14]. Carrier testing should be offered when the individual is assessed as having the capability of understanding the

process of carrier testing and its outcome. There may also be a role for genotyping in predicting the outcome of immune tolerance induction and for future gene-based hemophilia therapies [15]. In VWD, making a diagnosis can be challenging because while an objective personal history of excessive mucocutaneous bleeding can usually be obtained from the patient, incomplete penetrance and variable expressivity can make the documentation of a family history of the disease difficult, and laboratory tests of hemostasis can be variable in their ability to reveal either a quantitative or qualitative defect of von Willebrand factor (VWF). Furthermore, there is a lack of international consensus about diagnostic criteria for VWD. Molecular diagnostics is also important for the distinction of bleeding disorder genocopies, conditions with similar clinical presentations but different underlying genetic causes. Correct diagnoses of these conditions are an important component of clinical management as they may inform treatment decisions. With these issues as background, this review will consider the role of molecular genetic analysis as a complementary diagnostic modality for hemophilia and VWD, particularly where existing clinical and laboratory approaches to diagnosis have failed to provide a definitive answer.

3. Testing methodologies and quality assurance

Molecular testing for hemophilia A, hemophilia B, and von Willebrand disease is performed at many accredited and research-based facilities worldwide. A 2013 survey of international hemophilia care centers indicated that genetic diagnosis is available to approximately 75% of facilities, however, the proportion of patients who receive a genetic diagnosis is highly variable [16]. Testing methodologies for inherited bleeding disorders can also vary between facilities. Historically, conformation sensitive gel electrophoresis (CSGE) and linkage analysis by restriction length polymorphisms were routinely utilized,

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while some labs performed screening using denaturing high-performance liquid chromatography.

Currently, direct gene sequencing either through Sanger or next generation sequencing (NGS) methodologies predominates for the testing of single nucleotide variants [16]. Typically, these protocols involve sequencing the entire coding region of the gene, intron–exon boundaries, and in some cases additional regulatory regions. As Sanger sequencing is a costly and lengthy process, particularly of the large *F8* and *VWF* genes, many labs are adapting NGS protocols that allow for the simultaneous sequencing of multiple coagulation factor genes involved in both common and rare bleeding disorders [17,18]. Other commonly occurring variants such as deletions, duplications, and inversions can be assessed using complementary technologies. Copy number variant (CNV) analysis for large deletions and duplications can also be performed using NGS, array comparative genomic hybridization (aCGH), and multiplex ligation-dependent probe amplification (MLPA) [19–21]. 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 most frequently utilized [22–24].

As the availability and range of methodologies for genetic diagnosis for hemophilia and VWD continues to increase, the role of external quality assessment (EQA) programs for the evaluation of genotyping accuracy, standardization of nucleotide and protein variant descriptions using Human Genome Variation Society (HGVS) nomenclature, and variant pathogenicity assessment strategies have become essential. The UK National External Quality Assessment Scheme (UK NEQAS) Molecular Genetics of Heritable Bleeding Disorders scheme offer standardized external proficiency testing to international molecular diagnostic facilities for hemophilia A, B, and von Willebrand disease [25]. EQA allows for the standardization of the interpretation of genetic testing in the context of the patient's clinical phenotype and bleeding history [26]. This process can also provide guidance to ensure that reports are comprehensive for clinicians inexperienced with molecular genetics and genetic counseling [25].

4. Hemophilia A

Hemophilia A (HA) is an inherited bleeding disorder caused by dysfunctional, reduced or absent levels of the essential coagulation

cofactor, factor VIII (FVIII). HA is an X-linked recessive disorder, with a prevalence of 1/5000 live male births [1] (Table 1). Rare cases of HA in females result from inheritance of homozygous or compound heterozygous alleles or from chromosomal abnormalities. In these cases, loss of heterozygosity, Turner's syndrome (XO) and Testicular Feminization syndrome may warrant investigation [27]. Heterozygous female hemophilia carriers can have symptomatic bleeding associated with partial quantitative FVIII deficiency that may be influenced by skewed inactivation of the X chromosome [28]. HA is classified into mild (6–49% FVIII), moderate (1–5% FVIII), and severe presentations (<1% FVIII), with mild/moderate cases displaying bleeding associated with trauma or surgery, and severe cases presenting spontaneous muscle and joint bleeding.

HA is monogenic disorder resulting from pathogenic variants that occur in the *F8* gene that encodes the FVIII protein. The *F8* gene is 187 kb in size and is located on the long arm of the X chromosome at the most distal band (Xq28) (Fig. 1A) [10]. *F8* is comprised of 26 exons ranging in size from 69 bp (exon 26) to 3.1 kb (exon 14) that code for a 9 kb mRNA transcript. The *F8* gene has been traditionally described as monomorphic with only five nonsynonymous haplotypes occurring at a frequency of >1% in the normal population [29]. However, recent analysis of the whole *F8* gene in over 2500 normal individuals has reported a variety of rare and presumably benign *F8* gene variants located throughout this locus, and suggested an ethnic predisposition where Africans have an increased variant frequency compared with non-African subjects [30].

4.1. HA mutational spectrum

HA displays allelic heterogeneity, with pathogenic variants identified throughout the gene, although they can cluster in the A1, A2, and A3 domains. The majority of pathogenic HA variants can reduce the synthesis or secretion of FVIII, or impair FVIII cofactor activity. A small subset of variants localized to the A3, C1 and C2 domains are associated with impaired binding to the FVIII carrier molecule VWF [31], resulting in accelerated proteolysis and/or clearance of FVIII from the plasma. To date, more than 2000 unique *F8* gene variants have been reported in the FVIII Variant Database (Fig. 1B) [32]. The majority of HA cases are associated with single nucleotide variants (SNVs) (66.5%) (Fig. 2). Of these SNVs, missense mutations are most frequent across all disease

Table 1
Genetic characteristics of hemophilia A, B, and VWD subtypes.

Disorder	Prevalence	Mechanism	Variant location by protein domain	Variant location by exon	Variant types	Inheritance Pattern
Hemophilia A	1/5000 males	Quantitative and qualitative FVIII deficiency	<i>F8</i> whole gene	1–26	Point mutations, insertions, deletions, inversions	X-linked recessive
Hemophilia B	1/30,000 males	Quantitative and qualitative FIX deficiency	<i>F9</i> whole gene	1–8	Point mutations, insertions, deletions	X-linked recessive
Type 1 VWD	65–80%	Partial VWF deficiency (VWF:Ag 0.05–0.5 U/mL)	VWF whole gene, external loci?	1–52, promoter	Point mutations, insertions, deletions	Autosomal dominant, occasional recessive or co-dominant
Type 1C VWD	~15% of type 1	Accelerated VWF clearance from plasma	VWF D'D3, D4	18–27, 33–38	Missense variants	Autosomal dominant
Type 2A VWD	10–20%	Impaired multimerization	VWF propeptide, D'D3, A1, CK	2–28, 51, 52	Missense variants	Autosomal dominant
Type 2B VWD	5–10%	Gain-of-function binding to platelet GPIb	VWF A1	28	Missense variants	Autosomal dominant
Type 2M VWD	3–5%	Impaired platelet GPIb or collagen binding	VWF A1, A2, A3	28–32	Missense variants	Autosomal dominant
Type 2N VWD	2–5%	Decreased FVIII binding to VWF	VWF D'D3, propeptide cleavage site (2N), whole gene (null)	17–27 (2N) whole gene (null variant)	Missense variants, null variants (compound heterozygous)	Autosomal recessive homozygous, compound heterozygous
Type 3 VWD	1/1,000,000	Severe VWF deficiency (VWF:Ag < 0.05 U/mL)	VWF whole gene	1–52	Point mutations, insertions, deletions	Autosomal recessive, co-dominant

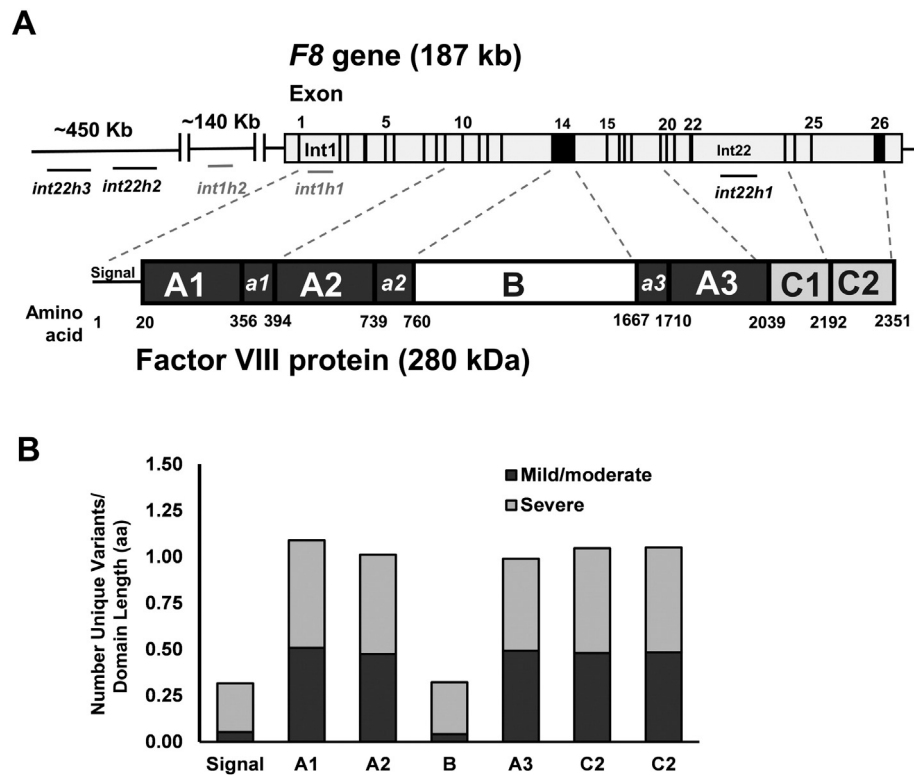


Fig. 1. Genetic testing for Hemophilia A. (A) *F8* gene and FVIII protein structure adapted from Graw et al. [116]. *Int1h* and *Int22h* = recombination sites. (B) Unique pathogenic variants reported by protein domain.

Data from: <http://www.factorviii-db.org>, accessed June 2016.

severities, with nonsense and splice site variants more frequently associated with severe HA. The remaining unique pathogenic variants associated with HA include deletions (23%), duplications (4.8%), insertions (1.6%) and indels (1.3%). Severe HA is associated with a higher proportion of deletions than mild/moderate HA. For mild/moderate and severe HA, the majority of pathogenic variants are localized to the A and C domains, although for severe HA, approximately 35% of nonsense variants are localized to the B domain.

4.2. Intron 22 and intron 1 inversions

Approximately 45% of severe HA is accounted for by an intrachromosomal inversion at intron 22, the result of a spontaneous

recombination event between a sequence within the *F8* intron 22 and one of two homologous sequences located approximately 450 kb upstream of the *F8* gene (Fig. 1A) [23,33]. This inversion event occurs spontaneously during meiosis in male germ cells [34]. A similar inversion event occurs between a homologous sequence within intron 1 that also occurs 140 kb upstream of the *F8* gene and accounts for a further 1–2% of severe HA cases [35]. The routine order of testing for HA variants is stratified based on disease severity, where severe cases first typically undergo intron 1 and intron 22 inversion analyses prior to sequencing or CNV analysis.

4.3. Mutation negative cases

Currently, routine molecular diagnostic protocols are able to identify pathogenic variants for approximately 95% of HA cases [36]. The missing 5% may be in part due to phenotypic misdiagnosis, as clinical presentation of HA is similar to its genocopy disorder type 2N VWD; as such molecular testing for type 2N VWD is recommended for all mild/moderate mutation negative HA cases. Tissue or germline mosaicism may also account for rare cases of variant negative HA, however, this would not be associated with patients with a family history. Additionally, rare cases of combined FV/FVIII deficiency are associated with pathogenic variants in the genes *MCDF2* (multiple coagulation factor deficiency 2) and *LMAN1* (lectin, mannose binding 1), which mediate the secretion of FVIII and FV from endothelial cells and hepatocytes, respectively [37]. Pathogenic HA variants may also be found outside the coding region of the *F8* gene, as recent mRNA analysis and whole *F8* gene analyses using NGS of mild and moderate variant negative HA cases have identified deep intronic variants that presumably alter mRNA splicing [38–40].

4.4. Immune response to FVIII

Complicating treatment for HA is the propensity for the development of FVIII-neutralizing antibodies or inhibitors in response to FVIII

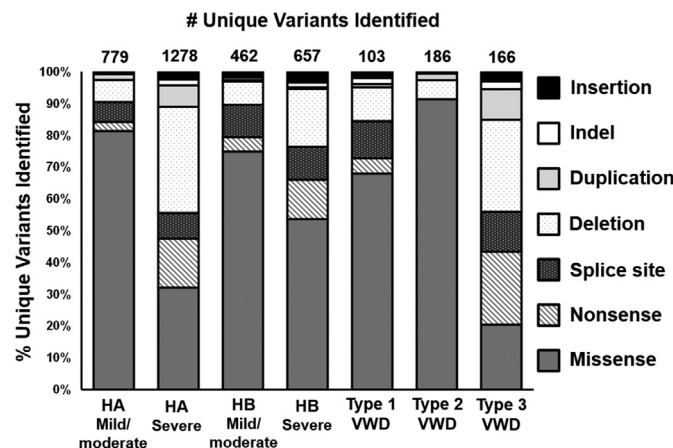


Fig. 2. Types of pathogenic variants in hemophilia and VWD. The frequency of mutation types across mild/moderate and severe HA cases, and VWF subtypes. Data accessed from the FIX variant database [103], accessed June 2016.

replacement products, a condition that affects between 5 and 30% of patients. The immune response to FVIII can be regulated by a number of environmental and genetic risk factors, and genetic variability within the *F8* gene, as well as at external loci that modify the anti-FVIII immune response can influence this risk. The incidence of inhibitor development is associated with disease severity, whereby mild/moderate HA patients have a decreased risk (3–13%) compared with severe HA patients (25–30%). This is likely related to the presence or absence of cross-reactive FVIII protein, where mild/moderate cases associated with missense variants (<10%) and small insertions/deletions may have a greater amount of circulating *F8* gene product [41], although a subset of missense variants that predispose to inhibitor development in non-severe HA [42]. Conversely, FVIII null mutations due to large deletions and nonsense variants associated with severe HA and are most likely to increase incidence of inhibitors (~75%) [41]. Intriguingly, the intron 22 inversion is associated with a lower risk for inhibitors as compared with other severe HA pathogenic variants; this may be related to the intracellular synthesis but not secretion of the entire *F8* gene product [43]. *F8* mutation status can also predict patient response to immune tolerance induction (ITI) therapy for inhibitor eradication, with large deletions associated with a decreased rate of ITI success [44].

Other genetic modifiers of FVIII inhibitor risk include HLA class II complex variants, and common single nucleotide variants in immunoregulatory genes including *IL10*, *TNFA*, *CTLA4*, and *FCGR2A* [45–48]. Recent whole exome sequencing of HA patients observed the presence of rare variants in additional regulatory genes that confer both protective and pathogenic influences on inhibitor incidence [49]. Incorporation of this knowledge into an algorithm for routine clinical testing may eventually become possible for this multigenic trait.

5. Hemophilia B

Hemophilia B (HB) is an inherited bleeding disorder caused by quantitative or qualitative defect of the coagulation zymogen factor IX (FIX). Like HA, HB is an X-linked recessive disorder, with a prevalence of 1/30,000 live male births, and a recognized bleeding phenotype in a subset of carriers (Table 1) [1,28]. The reduced incidence of HB relative to HA is likely related to the smaller size of the *F9* gene relative to *F8* (34 kb vs 187 kb), as well as to the absence of frequent inversion events associated with severe HA. Approximately 50% of HB cases are sporadic, without a known family history [26]. HB is associated exclusively with pathogenic variants located in the *F9* gene which encodes the FIX protein. *F9* is located on the long arm of the X chromosome (Xq27), is comprised of 8 exons and encodes an mRNA 2.8 kb in length (Fig. 3A) [50].

5.1. HB mutational spectrum

Pathogenic variants occur throughout the *F9* gene including the promoter and 3'UTR and are heterogeneous in nature. The FIX Variant Database currently reports over 1000 unique variants associated with this phenotype [32]. The majority of variants reported are single nucleotide substitutions (73%), with missense variants comprising a larger proportion of mild/moderate HB cases, and nonsense variants associated predominantly with severe HB (Fig. 2). Additionally, deletions (16.3%), insertions (3.3%), indels (1.5%), and duplications (0.4%) are also reported. In contrast to HA, no gene inversions have been associated with HB. The majority of variants localize to exon 8 (56%), which is the largest in size and encodes the functional serine protease domain of the active FIX coagulation factor (Fig. 3B).

5.2. Hemophilia B Leyden

HB Leyden (~2% of HB cases), is characterized by an initial presentation of severe or moderate FIX deficiency followed by postpubertal normalization of FIX levels [51]. This type of HB is associated with variants in the *F9* proximal promoter that disrupt binding sites to one of three

transcription factors — HNF (hepatic nuclear factor 4a), C/EBP (CCAAT enhancer-binding protein), or ONECUT1/2 (Fig. 3C) [52–54]. Phenotypic recovery is related to hormone-mediated activation of an androgen response element or growth factor activity which increases *F9* gene expression [55]. Sequencing the promoter proximal region of the *F9* gene is a routine part of the molecular diagnostic protocol for HB, and can be predictive of long-term normalization of FIX levels, although rare cases where phenotypic recovery does not occur have been documented [56].

5.3. Mutation negative HB cases

Similar to HA, the rate of pathogenic variant identification for HB is typically greater than 95%. Rare cases of multiple vitamin K-dependent coagulation factor deficiency (VKCFDs) involving prothrombin, FVII, FIX, and FX may be misdiagnosed as HB [26,37]. VKCFDs are associated with variants in *VKOR* (Vitamin K epoxide reductase) or *GGCX* (γ -glutamyl carboxylase) which mediate the γ -carboxylation of the Gla-domain on these coagulation factors [57]. Deep intronic variants in the *F9* gene have also been identified for some cases of HB [58]. Additionally, for some mutation negative mild/moderate HB families, silent single nucleotide variants have been identified [59]. These variants may influence splicing by altering splice enhancer or repressor recognition motifs. Codon alterations may also influence transcription or translational mechanisms by modulating the stability and structure of the *F9* mRNA, rate of translation, or result in misfolding of the protein. For example, the synonymous variant c.459G>A (p.V153=) is reported to decrease expression and altered FIX protein conformation [60]. Additional functional studies are required to better characterize the influence of these variants on the production and activity of the FIX protein and confirm pathogenicity.

5.4. Immune response to FIX

The incidence of inhibitors is significantly lower in HB (between 1.5 and 3%) than in HA. This may in part be related to the increased proportion of HB patients that express cross-reactive antigen compared to HA. Inhibitors in HB will present most frequently in patients with nonsense variants and large deletions; less frequently small insertions or deletions are reported to associate with inhibitors, and only rare instances of missense variants associated with inhibitors are reported [61,62]. Almost 60% of HB patients that develop inhibitors will also experience an anaphylactic response to FIX concentrate at the time of inhibitor onset; this complication is also more likely to be associated with large deletions or null mutations than SNVs [62].

6. von Willebrand disease

von Willebrand disease is the most common inherited bleeding disorder, with prevalence estimates ranging from 0.1 to 1% [2–4]. It is characterized by excessive mucocutaneous bleeding and caused by quantitative or qualitative abnormalities of von Willebrand factor, a multimeric glycoprotein that plays essential hemostatic roles in the circulation. VWD exhibits autosomal inheritance, with Types 1, 2A, 2B and 2M being primarily dominant and 2N and Type 3 VWD being primarily recessive (Table 1). The *VWF* gene was cloned in 1985 by four groups in the US and Europe. The gene is located on the short arm of chromosome 12 at the locus 12p13.3 [5–7,9]. It spans approximately 178 kb and contains 52 exons that range in size from 1.3 kb (exon 28) to 40 bp (exon 50) (Fig. 4A) [63].

6.1. VWD mutational spectrum

6.1.1. Type 1 VWD

Type 1 VWD, which is characterized by a partial deficiency of qualitatively normal VWF, represents the most common form of the disease (~65–75% of all VWD cases) but is the most challenging to diagnose. Five

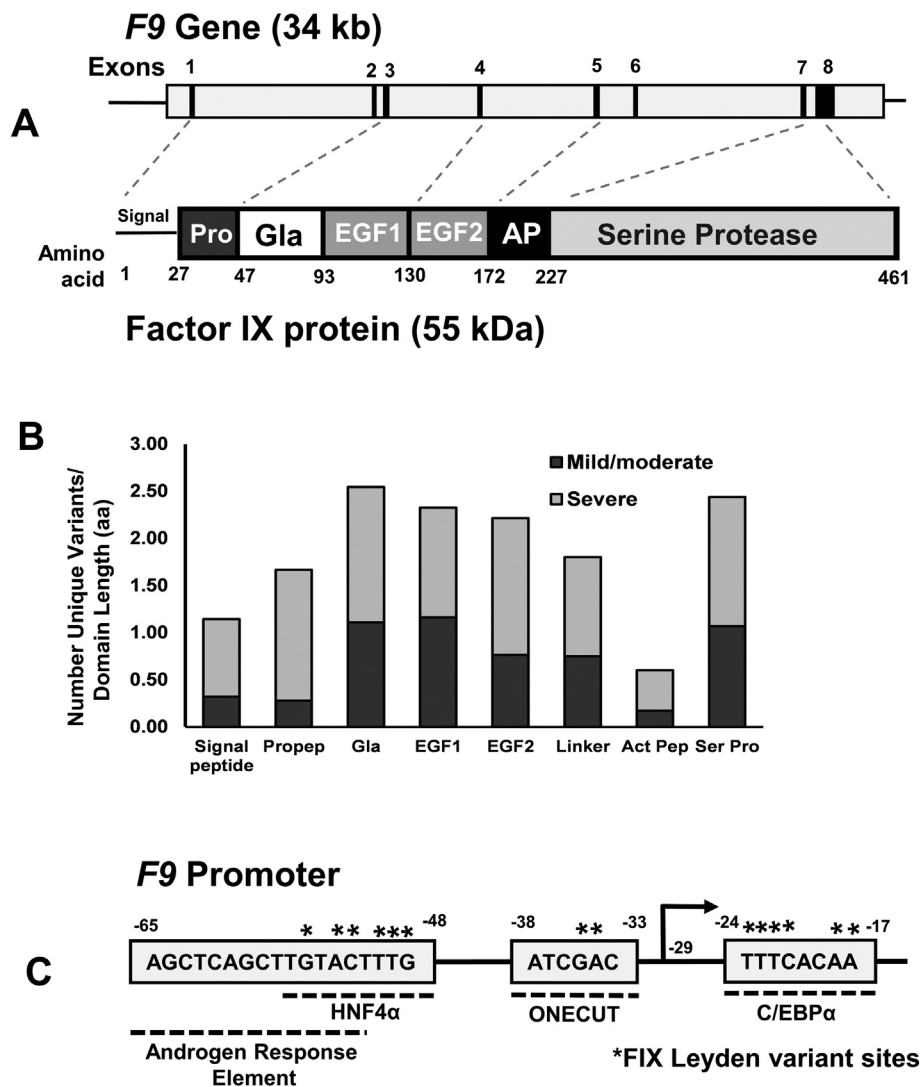


Fig. 3. Genetic testing for Hemophilia B. (A) F9 gene and FIX protein structure. (B) Unique pathogenic variants reported by protein domain. Data from: <http://www.factorix.org>, accessed June 2016. (C) Sites of Hemophilia B Leyden variants adapted from Funnell and Crossley [51].

large multicenter studies have evaluated the genetic basis of Type 1 VWD and have reported consistent results on >600 families [64–68]. Mutations in the *VWF* gene coding region, promoter and splice sites (predominantly missense mutations) (Fig. 4B) were identified in ~65% of index cases and were found more frequently, and with higher penetrance, in cases with lower VWF levels. The most frequently reported genetic variation (10–20% of index cases) identified in all studies was a missense mutation resulting in an amino acid substitution of tyrosine to cysteine at codon 1584 (Tyr1584Cys) [69]. Investigation is ongoing as to the pathogenicity and underlying mechanism of this variant. In an ongoing large US study, the frequency of non-pathogenic *VWF* sequence variation was highlighted, with particular variants showing a racial predisposition, potentially contributing to false positive diagnoses in certain populations [70–72]. Additionally, sequence variants of uncertain significance are frequently identified, and in ~35% of Type 1 VWD patients no obvious *VWF* mutation is found. Studies are ongoing to determine the cause of low VWF in these cases. These recent studies have therefore confirmed prior thinking that type 1 VWD represents a complex genetic trait, manifesting both allelic and locus heterogeneity. This genetic complexity makes the use of molecular genetic testing as a complementary diagnostic aid in the majority of Type 1 VWD cases questionable at the present time. Type 1C VWD is not currently recognized in the ISTH Classification, but does have clinical importance as the underlying pathophysiology involves increased VWF clearance

which limits the effectiveness of desmopressin. Specific *VWF* mutations have been identified that affect VWF clearance [73,74].

6.1.2. Type 2 VWD

Type 2A VWD accounts for ~10% of all VWD cases and is characterized by decreased VWF–platelet binding as the result of the loss of high and intermediate molecular weight multimers. Type 2A VWD has been associated with more than 50 different missense mutations that act through a number of mechanisms, often in combination: 1) enhanced susceptibility to ADAMTS13-mediated proteolysis, 2) impaired dimer and multimer assembly, 3) loss of regulated storage and 4) intracellular retention [75–77]. In addition to providing further insights into VWF structure/function, genetic testing for type 2A VWD can be employed when phenotypic uncertainty exists. Type 2A VWD mutations occur most frequently in the A2 domain but are also found in other domains involved in multimerization including D'D3, CK, and the VWF propeptide (D1 and D2) (Fig. 4C).

Type 2B VWD is the result of 'gain-of-function' missense mutations within the GpIb binding site of VWF. This leads to spontaneous VWF–platelet interactions that result in the selective depletion of high molecular weight (HMW) multimers and subsequent thrombocytopenia. VWF binding to platelets can also lead to enhanced ADAMTS13-mediated proteolysis, which contributes to the loss of HMW multimers [78,79]. Although reports of its safe use exist in some Type 2B VWD

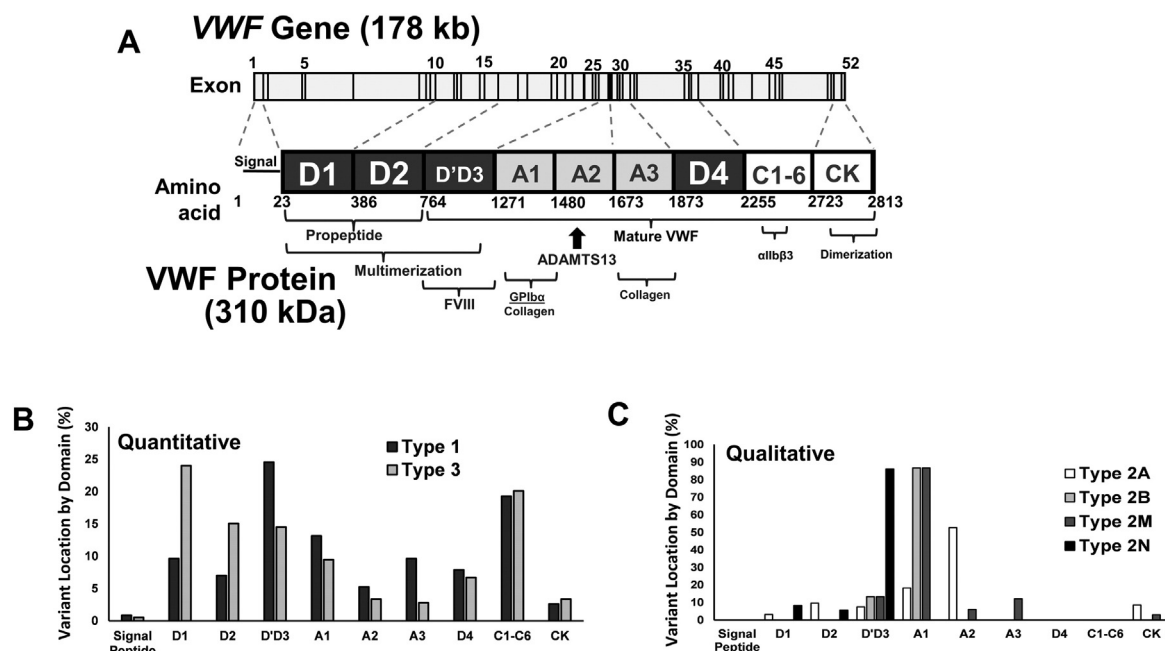


Fig. 4. Genetic testing for von Willebrand disease. (A) VWF gene and VWF protein structure. (B) Unique pathogenic variants reported by protein domain (type 1 and type 3 VWD). (C) Unique pathogenic variants reported by protein domain (type 2 VWD). Data from: <http://www.vwf.group.shef.ac.uk/vwd.html>, accessed June 2016.

cases, desmopressin is generally considered relatively contraindicated in these patients because of the risk of worsening thrombocytopenia, therefore, the diagnosis of Type 2B VWD is of therapeutic importance. Genetic testing can be helpful in this regard, particularly if interpretation of phenotypic assays is difficult.

Type 2M VWD is classically characterized by decreased VWF-platelet interactions; however, in contrast to Type 2A, multimerization is normal. Causative mutations have been localized to the platelet GPIIb binding site, in the A1 domain of VWF, although at distinct locations from Type 2B mutations [80]. Type 2M VWD mutations have also been reported in the A3 and A1 domains, resulting in decreased VWF binding to subendothelial collagen [81,82]. Genetic testing can be helpful, although the main therapeutic consequence of Type 2M is a poor clinical response to desmopressin, which can usually be identified through a therapeutic trial [83].

Type 2N VWD was first described as an autosomal form of hemophilia A and is an important differential in the investigation of all individuals (male and female) presenting with a low FVIII level [84]. The ease of analysis of exons 17–25 of the VWF gene and the relative lack of widespread availability of standardized FVIII binding assays has increased interest in using genetic testing to confirm this diagnosis. While types 2A, 2B, and 2M VWD are almost always inherited in an autosomal dominant manner, type 2N VWD is inherited in a homozygous or compound heterozygous manner, involving either two 2N alleles or a 2N and a VWF null allele.

6.1.3. Type 3 VWD

In most instances, the severe clinical phenotype, absent plasma VWF, and very low factor VIII:C (<0.10 U/mL) levels make the diagnosis of Type 3 VWD straightforward. Despite this, parents of type 3 VWD individuals may be interested in genetic testing for future family planning purposes and mutation detection can provide definitive information that can be utilized for prenatal testing. Recent studies indicate that in addition to the classical recessive pattern of inheritance, some type 3 VWD kindreds demonstrate co-dominant inheritance [85]. Type 3 VWD has a heterogeneous mutational basis with more than 80 different mutations having been described to date including VWF gene insertions, nonsense and missense mutations as well as partial and total VWF gene

deletions (Figs. 2, 4B) [85–87]. In addition to its use in the setting of family counseling, especially for prenatal diagnosis, VWF genotyping may also be of value with regard to predicting the rare possibility of anti-VWF alloantibody development following exposure to therapeutic concentrates [88].

6.2. Mutation negative cases

As mentioned, in ~35% of Type 1 VWD patients, no obvious VWF mutation was identified. These are usually cases at the milder end of the spectrum, and it is possible that the genetic determinants of low VWF are likely to be more complex and could involve either intronic sequence variants and/or variants at other genetic loci [89]. Certainly, it is well recognized that blood group O predominates in this group of patients and possibly influences VWF levels through clearance mechanisms [90,91]. The identification of novel loci that can modulate plasma VWF levels in normal individuals has been the subject of several recent genome-wide association studies (GWAS) and linkage analyses [92,93]. Preliminary studies suggest that several of these novel variants can modify the type 1 VWD phenotype [89,94], and there is a long-term goal of incorporating these analyses into a diagnostic algorithm for this multigenic trait.

Additionally, genetic testing can definitively distinguish Type 2B VWD and platelet-type (PT)-VWD, which is caused by 'gain-of-function' mutations in the *GPIBA* gene [95]. This is a critical distinction as the mainstay of treatment in Type 2B VWD is VWF replacement while in PT-VWD it is platelet transfusion therapy. Finally, the distinction between Type 2N VWD and hemophilia A is important, as the treatments are different (type 2N VWD may respond to desmopressin or will require infusion of a FVIII/VWF concentrate as opposed to a FVIII concentrate, which is used in hemophilia A), and the patterns of inheritance are different. Genetic analyses of both the VWF and *F8* genes can easily determine the diagnosis.

6.3. Immune response to VWF

The development of alloantibodies against VWF represents a rare but serious complication of treatment of VWD, occurring in ~5–10% of

type 3 VWD patients [96,97]. Affected patients can present with a range of symptoms including lack or loss of hemostatic response to infused VWF concentrates up to anaphylactic reactions in rare cases [88]. It is classically reported in multi-transfused patients, and occurs most frequently in patients with partial or complete VWF gene deletions [86, 98]. A positive family history of anti-VWF antibodies also appears to be a risk factor. There is a lack of standardization of laboratory methods for antibody identification and characterization which is a barrier to future studies. Recombinant factor VIII, as well as bypassing agents and immune tolerance have been reported as effective treatments, however aside from case reports, little exists in the literature to guide management [99,100].

7. Assessment of variant pathogenicity

Although genetic diagnosis for inherited bleeding disorders has been available for 30 years, understanding the association between an identified variant and a disease phenotype remains challenging. With the development of NGS sequencing protocols, the rate of identification of pathogenic variants associated with deficiencies in coagulation factors FVIII, FIX, and VWF will likely increase, as will our knowledge about the genetic diversity of these loci within the normal population. The *F8*, *F9*, and *VWF* genes are associated with polymorphisms that occur with a minor allele frequency of >1% of the population, as well as rare benign variants that do not associate with a disease phenotype. Therefore, the presence of a novel variant, even in association with a disease phenotype, is not sufficient evidence to impute pathogenicity.

Genetic diagnosis for the hemophilias and VWD can also be complicated by the incomplete penetrance and variable expressivity of these conditions. For example, ethnicity can influence the amount of diversity at the *F8* and *VWF* genes. Additionally, some VWD variants that are pathogenic in Caucasian populations appear to be benign for individuals with African ethnicity, potentially contributing to an increase rate of false positive diagnoses [71,72]. While this phenomenon is not currently well understood, it may be related to false attribution of variant pathogenicity in the initial characterization of these variants, or expressivity that may be influenced by both environmental and genetic factors. Moreover, the prediction of the interaction or additive effect of multiple polymorphic variants, or rare variants of low penetrance remains difficult.

While the influence of frame shift mutations, large rearrangements, large CNVs, and premature termination codons on disease phenotype are clearly pathogenic and typically do not require extensive analysis, systematized protocols involving multiple lines of evidence are required for the characterization of missense and splice site associated variants. The American College of Medical Genetics (ACMG) [101] and Association for Clinical Genetic Science (ACGS) [102] provide standards for the clinical assessment of unclassified variants. The association of a variant with a disease phenotype in unrelated families can be queried in disease specific databases, including those maintained by the European Association for Hemophilia and Allied Disorders (EAHAD) (Table 2) [103]. These sites aggregate structural and clinical information

associated with each variant and document the number of cases where the variant has been associated with a disease phenotype. The presence of a variant in a reference sequencing database of normal individuals can be evidence of non-pathogenicity, particularly when supported by unequivocal allele frequency data. These resources include the 1000 genomes, ExAC, dbSNP, and NHLBI exome sequencing project (Table 2). Additionally, pedigree analysis can be used to demonstrate the association of a variant with a disease phenotype within a given family, or the co-occurrence of a new variant with the sporadic presentation of a bleeding phenotype within the family.

Assessment of the influence of identified variants on protein production and function can be performed using both cell and animal-based models. Heterologous in vitro systems can assess the influence of candidate variants on mRNA synthesis, stability and splicing, as well as protein synthesis, storage, secretion, and structure [60,104,105]. Animal models can be utilized to characterize protein production, half-life, and the hemostatic efficacy of variant proteins using models of hemostasis and thrombosis [106,107]. Recently, patient-derived endothelial progenitor cells have provided utility in the characterization of VWF gene variants and understanding genotype–phenotype associations [85,108,109]. Therefore, review of published literature for variant characterization studies should be a routine component of variant pathogenicity assessment.

7.1. Missense variants

In silico characterization of candidate missense variants is also an important component of pathogenicity assessment. For missense variants, a variety of programs are available for predicting the influence of nonsynonymous changes on protein function, including Polyphen-2, SIFT, and Mutation Taster [110–112]. These tools are developed based on different algorithm types including machine and supervised learning, evolutionary conservation of DNA and protein sequence, and structure analysis. For example, the evolutionary conservation of a given amino acid across multiple species is assessed, where missense substitutions at invariant sites are considered to be more pathogenic than substitutions at positions with less constraint. Assessment may also incorporate the Grantham score, a measurement of the relative change to the protein by an amino acid substitution, based on physiochemical properties including composition, polarity, and molecular volume [113]. Typically, missense assessment platforms will provide a prediction of the influence of the variant on protein function (e.g. benign or pathogenic) as well as a score indicating degree of confidence of that assessment. The accuracy of these assessments may be variable depending on the gene under investigation, but overall have an accuracy of approximately 65–80% [114]. Investigations utilizing in silico nonsynonymous variant assessment should include analysis from at minimum three programs of different algorithm types to reduce the possibility of reporting a false positive or false negative result. These tools are not considered to be definitive and additional lines of evidence must be considered in evaluating variant pathogenicity.

Table 2
Select variant registries.

Database	Location	Information	Disorder
FVIII variant database	www.factorviii-db.org	Variant registry	Hemophilia A
FIX variant database	www.factorix.org	Variant registry	Hemophilia B
von Willebrand factor variant database (VWFdb)	www.vwf.group.shef.ac.uk	Variant registry	VWD
ClinVar	www.ncbi.nlm.nih.gov/clinvar	Variant registry	Mendelian disorders
Human gene mutation database (HGMD)	www.hgmd.cf.ac.uk/ac/index.php	Variant registry	Mendelian disorders
1000 genomes project	www.1000genomes.org	Genomic variation	Normal individuals
Exome aggregation consortium	www.exac.broadinstitute.org	Exome sequence variants	Mendelian disorders, normal individuals
dbSNP	www.ncbi.nlm.nih.gov/SNP	Small genetic variations	Mendelian disorders, normal individuals
NHLBI exome sequencing project (ESP)	evs.gs.washington.edu/EVS	Exome sequencing data	Heart, lung and blood disorders

7.2. Splice site variants

Variants should also be assessed *in silico* for influence on mRNA splicing. While substitutions that alter the consensus dinucleotide splice site (+/−1, +/−2) may be considered pathogenic and require no additional investigations, missense, exonic synonymous, and intronic variants close to the intron–exon boundary may also either abolish existing or create novel splice sites. Platforms such as NNSplice, MaxEntScan, and Human Splice Finder have a good degree of accuracy for variants between positions +7 and −10 [115]. As for missense analysis, the use of 3 or more splice site tools guards against erroneous reports.

7.3. Reporting

ACMG and ACGS recommend systematic characterization of variants associated with Mendelian disorders on a standardized scale, as “Class 5 – Pathogenic (confirms diagnosis)”, “Class 4 – Likely pathogenic (consistent with diagnosis)”, “Class 3 – Uncertain significance (does not confirm or exclude diagnosis)”, “Class 2 – Likely benign (diagnosis not confirmed)” and “Class 1 – Benign (diagnosis not confirmed)” based on the strength of the associated evidence. Evidence for Class 5 and Class 4 pathogenicity is based on the number of independent observations that associate a variant with a disease phenotype, as well as functional characterization of variant pathogenicity in the published literature. Guidelines suggest reporting only Class 4 and 5 variants, or Class 3 variants particularly when no other pathogenic candidates are observed [102].

8. Conclusion

Access to molecular diagnostic testing for hemostatic disorders is increasing in recent years, although at present this expertise is not typically available in routine clinical hemostasis laboratories. Our level of understanding of the molecular genetic aspects of hemophilia and VWD makes the inclusion of this knowledge appropriate in routine clinical care. Additionally, the application of novel advances in genomic analysis including genome-wide linkage analysis and whole genome or whole exome sequencing will allow for the detection of loci responsible for bleeding in individuals without diagnosis, ultimately improving the care of patients with inherited bleeding disorders. The potential to incorporate genetic information into possible therapeutic approaches is an exciting prospect for the future.

Practice points

- Genetic diagnoses for unspecified or undiagnosed bleeding disorders are inappropriate and should be avoided.
- Genetic diagnoses for inherited bleeding disorders should be utilized as a complementary diagnostic modality.
- Carrier testing should be utilized when potential female carriers begin family planning or become pregnant, or are capable of understanding the process of carrier testing and its outcome.
- Genetic diagnoses for inherited bleeding disorders may be utilized to provide clarification when clinical symptoms are unclear and distinguish bleeding disorder genocopies.

Research agenda

- Characterization of the effects of synonymous variants on protein function
- Improved pathogenicity prediction for nonsynonymous substitutions
- Whole gen(ome) sequencing for variant negative cases
- Association of variants at novel genetic loci with type 1 VWD
- Association of variants at immunoregulatory genes with immune response to FVIII, FIX

- Increased understanding of mechanisms that influence variant penetrance and expressivity.

Conflict of interests

The authors have no relevant conflicts to disclose.

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