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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.



## 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 | *F8* whole gene | 1–26 | Point mutations, insertions, deletions, inversions | X-linked recessive |
| Hemophilia B | 1/30,000 males | Quantitative and qualitative FIX deficiency | *F9* 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 |