

# 4 GENETIC ASSESSMENT

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**All statements identified as recommendations are consensus based, as denoted by **CB**.**

## 4.1 | Introduction

- Genetic assessment of hemophilia is important in defining disease biology, establishing diagnosis in difficult cases, predicting risk of inhibitor development, identifying female carriers, and providing prenatal diagnosis, if desired.<sup>1</sup>
- Genotype analysis should be offered to all people with hemophilia and their “at-risk” female family members.
- Genetic testing strategies are led by the phenotypic parameters measured by the coagulation laboratory in addition to the family pedigree. Therefore, it is essential that the data are made available to the genetic testing laboratory. An accurate interpretation of the underlying variant(s) detected is dependent on the supporting phenotypic data and family history for the patient.<sup>2-5</sup>
- Genetic counselling for people with hemophilia and their families is an essential requirement prior to genetic testing. This includes obtaining informed consent from the patient, parent, or legal guardian, requiring both permission to carry out testing as well as education to ensure that they fully understand the testing procedure, the benefits and limitations of the test, and possible consequences of the test results.<sup>6,7</sup>
- Genetic counselling should also provide information and advice about prenatal diagnosis (PND), management of pregnancy and delivery in hemophilia carriers, and pre-implantation genetic diagnosis (PGD). It is important to be aware of and follow the relevant laws governing such procedures in the country where the service is being provided.
- Genetic testing will not always identify the underlying variant associated with the hemophilia phenotype. Genetic counselling should highlight this possibility to the individual referred for genetic testing. (See Chapter 9: Specific Management Issues – Carriers – Genetic counselling – Psychosocial support.)
- Genetic diagnostic laboratories should adhere to strict protocols and procedures, which require:
  - knowledge and expertise in genetic laboratory testing;
  - use of the correct investigative platforms;
  - knowledge and expertise in the interpretation of the genetic variants identified in association with hemophilia;
  - use of the correct interpretative platforms for investigation of variants;
  - use of the correct nomenclature for description of variants and the correct classification systems for determining pathogenicity of variants;
  - internal quality control procedures;
  - participation in periodic accreditation, where available; and
  - participation in external quality assessment schemes (EQAS), where available.
- The interpretation of the results of genetic testing should be performed by scientists who have knowledge and expertise in hemophilia genetics.
- The opportunity for discussion of the genetic results between the ordering clinician and reporting scientist is an essential provision of the genetic diagnostic service.

### RECOMMENDATION 4.1.1:

- For people with hemophilia, the WFH recommends that genetic testing be offered to identify the specific**

underlying genetic variant associated with their disorder. **CB**

#### RECOMMENDATION 4.1.2:

- For obligate carriers of hemophilia and “at-risk” female relatives of people with hemophilia or potential carriers of hemophilia, the WFH recommends that genetic testing be offered for the previously identified genetic variant in the *F8* or *F9* gene. **CB**

#### RECOMMENDATION 4.1.3:

- For females with low phenotypic coagulation FVIII or FIX levels, the WFH recommends that investigation of the genetic/epigenetic basis of the phenotype be offered. **CB**

#### RECOMMENDATION 4.1.4:

- For obligate carriers of hemophilia and “at-risk” female relatives of people with hemophilia or potential carriers of hemophilia, the WFH recommends the inclusion of a detailed family pedigree to support the genetic testing referral. **CB**

#### RECOMMENDATION 4.1.5:

- For individuals with suspected hemophilia and potential carriers of hemophilia, the WFH strongly recommends that phenotypic screening for FVIII or FIX levels, von Willebrand factor (VWF) antigen, and VWF activity testing be performed prior to referral for genetic testing. **CB**

#### RECOMMENDATION 4.1.6:

- For people with hemophilia, obligate carriers of hemophilia, “at-risk” female relatives, or individuals with low coagulation factor levels, the WFH strongly recommends detailed genetic counselling prior to offering genetic testing.
- **REMARK:** Genetic counselling should include a discussion of the experimental limits of the molecular results according to the availability of practical approaches.
- **REMARK:** Genetic counselling should include a discussion of the possibility of incidental findings in genes other than *F8* or *F9*, if the methodology used by the investigating laboratory (e.g., next generation sequencing [NGS]) may detect such genetic variations.
- **REMARK:** Genetic counselling should be performed by a genetic counsellor when available. If no genetic counsellor is available, a medical professional with

knowledge of genetics in hemophilia can provide genetic counselling. **CB**

#### RECOMMENDATION 4.1.7:

- For all patients referred for genetic testing, the WFH strongly recommends that informed consent be obtained from the patient, parent, or legal guardian. This requires both permission to carry out testing and education to ensure that they fully understand the testing procedure, the benefits and limitations of the test, and possible consequences of the test results.
- **REMARK:** Written informed consent may need to be obtained and documented by the clinician or genetic counsellor in compliance with local policies and practices. **CB**

## 4.2 | Indications for genetic assessment

- Genetic testing is generally sought in all affected cases (proband) and “at-risk” female relatives within the family.
- Ideally, the disease-causing variant should first be identified in the proband or the obligate carrier. All other potential carriers may subsequently be screened for this variant to confirm or exclude the carrier status.
- If neither the proband nor the obligate carrier are available for testing, the genetic assessment may still be performed in potential carriers; however, when a disease-causing variant is not detected, it should be clearly mentioned in the report that failure to detect genetic variants with the existing techniques does not exclude the carrier status.
- Carriers of hemophilia exhibit a wide range of factor levels, with approximately 30% having levels <40 IU/dL.<sup>8</sup> Women and girls with low or borderline levels can experience a range of bleeding symptoms, usually consistent with mild hemophilia, but hemarthrosis and more severe bleeding symptoms can occur.<sup>9,10</sup>
- Besides the heterozygosity for the disease-causing variant, low factor levels in carriers of hemophilia may be attributed to other epigenetic factors such as X-chromosome inactivation (XCI)<sup>11,12</sup> or the ABO blood group system.<sup>13</sup>
- Pregnant women who are confirmed carriers of an *F8* or *F9* variant may be offered non-invasive testing to determine the sex of the fetus they are carrying in order to inform subsequent options for prenatal diagnosis in a male fetus. This is achieved through analysis of cell-free fetal DNA in the maternal plasma.<sup>14-16</sup>
- Prenatal diagnosis may be offered to all confirmed carriers of an *F8* or *F9* variant who are carrying a male fetus in

early pregnancy by chorionic villus sampling or in late pregnancy by late-gestation amniocentesis, in order to guide the management of the delivery or to terminate the pregnancy in case of an affected fetus.<sup>17-20</sup> Genetic counselling should include a discussion of the risk of the PND procedure to the pregnancy.

- Pre-implantation genetic diagnosis may be offered to confirmed carriers of an *F8* or *F9* variant in order to select an embryo that will not result in the birth of a male with hemophilia.<sup>21,22</sup>
- It is important to be aware of and follow the relevant laws governing genetic counselling and pre-implantation genetic diagnosis in the country where the services are being provided.
- Among all the genetic risk factors, the nature of disease-causing variants in both *F8* and *F9* has been found to be the strongest risk factors for inhibitor development. Null variants, i.e., variants which result in total absence of the protein (large deletions, duplications, insertions, inversions, nonsense mutations, and splice-site variants), have shown the strongest association with inhibitors as compared to other variants (small in-frame deletions, duplications, insertions, missense mutations).<sup>23-33</sup> The response to immune tolerance induction (ITI) therapy has also been reported to be associated with the disease-causing variants with the latter group showing good response to ITI as compared to patients carrying null variants.<sup>34</sup>
- Some of the gene manipulation techniques (e.g., nonsense mutation suppression and gene editing) may require prior information of the disease-causing variants.
- Genetic assessment may be offered to:
  - all cases with clinically suspected hemophilia or hemophilia cases with confirmed laboratory diagnosis;
  - all obligate carriers to identify the molecular variant for possible future prenatal diagnosis;
  - all at-risk female family members to establish carrier status, which is critical for optimal prenatal counselling and testing if indicated, or to offer pre-implantation genetic diagnosis;
  - all symptomatic females (with low FVIII or FIX levels) with no family history;
  - predict the risk of inhibitor development in individuals with hemophilia;
  - predict the response to ITI therapy;
  - ascertain the feasibility of some gene manipulation techniques.
- See Chapter 3: Laboratory Diagnosis and Monitoring.

#### **RECOMMENDATION 4.2.1:**

- For people with suspected or established hemophilia undergoing genetic testing, the WFH recommends that the index case (pro-band) be genotyped to identify the underlying genetic variant. **CB**

#### **RECOMMENDATION 4.2.2:**

- For obligate carriers of hemophilia and “at-risk” female relatives of the affected proband or potential carrier of hemophilia, the WFH recommends genetic counselling about their risk of being a carrier. **CB**

#### **RECOMMENDATION 4.2.3:**

- For all obligate carriers of hemophilia and “at-risk” female relatives of people with hemophilia or potential carriers of hemophilia, the WFH recommends that phenotypic coagulation factor levels be measured. **CB**

#### **RECOMMENDATION 4.2.4:**

- For all obligate carriers of hemophilia and “at-risk” female relatives of people with hemophilia, the WFH recommends that genetic testing be offered for the previously identified genetic variant in the *F8* or *F9* gene. **CB**

#### **RECOMMENDATION 4.2.5:**

- For females with low phenotypic coagulation FVIII or FIX levels, the WFH recommends that investigation of the genetic/epigenetic basis of the phenotype be offered. **CB**

#### **RECOMMENDATION 4.2.6:**

- For pregnant females who are carriers of an *F8* or *F9* variant and are carrying a male fetus, the WFH recommends that prenatal diagnosis (PND) be offered to determine the hemophilia status of the fetus.
- **REMARK:** Genetic counselling should include a discussion of the risk of the PND procedure to the pregnancy.
- **REMARK:** It is important to be aware of and follow the relevant laws governing such procedures in the country where the service is being provided. **CB**

#### **RECOMMENDATION 4.2.7:**

- For families who wish to be prepared for a child with hemophilia before birth or who wish to terminate an affected fetus, the WFH recommends that prenatal diagnosis (PND) by chorionic villus sampling or amniocentesis be offered.

- **REMARK: It is important to be aware of and follow the relevant laws governing such procedures in the country where the service is being provided.**
- **REMARK: PND may be offered in early pregnancy or in late pregnancy by late-gestation amniocentesis in order to guide the management of the delivery of an affected child. **CB****

#### **RECOMMENDATION 4.2.8:**

- For people with suspected or established hemophilia, the WFH recommends that genetic testing be performed; knowledge of the genetic variant may help predict the risk of inhibitor development, response to immune tolerance induction (ITI), and depth of phenotype severity, as well as determine the availability of gene manipulation techniques. **CB**

### **4.3 | Strategy for genetic testing of probands**

- Worldwide, approximately 30-45% of patients with severe hemophilia A show an unusual type of structural variant (SV), a large DNA inversion affecting the *F8* intron 22 (i.e., the intron 22 inversion, Inv22).<sup>35,36</sup>
- The *F8* intron 22 inversion originates almost exclusively from male germ cells<sup>37</sup> by an event of homologous recombination between large inverted repeated sequences.<sup>38</sup> Reported evidence in the literature supports the fact that almost all mothers of patients with the Inv22 are carriers<sup>39</sup> and that the Inv22 is the most prevalent cause for severe hemophilia A worldwide.<sup>40-44</sup>
- A second recurrent inversion event causing approximately 2% of severe hemophilia A phenotypes worldwide is the *F8* intron 1 inversion (Inv1).<sup>45</sup>
- The remaining patients with severe, moderate, or mild hemophilia A (i.e., uninformative for the common *F8* inversions), as well as all patients with hemophilia B, generally have small variants in *F8* or *F9*, such as single nucleotide substitutions, small insertions, duplications or deletions, or, less frequently, large copy number variations (CNVs).
- Information about *F8* and *F9* variants is compiled in internationally accessible databases, such as those developed by the Centers for Disease Control and Prevention (CDC), named CDC Hemophilia A Mutation Project (CHAMP) and CDC Hemophilia B Mutation Project (CHBMP; <http://www.cdc.gov/ncbddd/hemophilia/champs.html>),

and by the European Association for Haemophilia and Allied Disorders (EAHAD) for *F8* and *F9*.

#### **RECOMMENDATION 4.3.1:**

- For male probands, the WFH recommends that genetic testing be directed by the proband's baseline phenotypic coagulation factor level, which indicates the severity of the disorder.
  - In patients with severe hemophilia A (FVIII:C <1 IU/dL) or moderate hemophilia A with lower-borderline factor activity levels (FVIII:C 1-3 IU/dL), analysis of the *F8* intron 22 inversion and the *F8* intron 1 inversion should be performed first.
  - Patients with severe hemophilia A in whom recurrent inversions (i.e., *F8* intron 22 and intron 1 inversions) cannot be detected should undergo screening and characterization of small variants, including single nucleotide variants (SNV) and small insertion, duplication, or deletion variants covering the essential regions of *F8* including the 26 exons, exon/intron boundaries, and 5' and 3' untranslated regions. If these tests are still uninformative, patients should be screened for copy number variants (CNV) including large *F8* deletions, duplications, or complex rearrangements.
  - In patients with moderate (FVIII:C 1-5 IU/dL) or mild (FVIII:C 5-40 IU/dL) hemophilia A, screening and characterization of small variants (i.e., SNV and small insertions, duplications, or deletions) covering the essential regions of *F8* including the 26 exons, exon/intron boundaries, and 5' and 3' untranslated regions should be performed first. If these tests are still uninformative, patients should be screened for *F8* CNV.
  - In all patients with hemophilia B (i.e., patients with severe [FIX:C <1 IU/dL], moderate [FIX:C 1-5 IU/dL], and mild [FIX:C 5-40 IU/dL] hemophilia B), screening and characterization of small variants (i.e., SNV and small insertions, duplications, or deletions) covering the essential regions of *F9* including the 8 exons, exon/intron boundaries, and 5' and 3' untranslated regions should be performed first. If these tests are still uninformative, patients should be screened for *F9* CNV. **CB**


#### 4.4 | Techniques for genetic assessment

- The *F8* gene is localized to the long arm of the X chromosome at Xq28. *F8* spans 187 kb of genomic DNA and consists of 26 exons encoding a mRNA of 9.0 kb. The mature FVIII protein has 2,332 amino acids.
- The *F9* gene is localized to the long arm of the X chromosome at Xq27. *F9* spans 33 kb of DNA and comprises 8 exons. *F9* mRNA is 2.8 kb and encodes a pre-pro-protein of 461 amino acids that is post-translationally processed to yield a mature protein of 415 amino acids.
- Different techniques (e.g., Southern blot, long-range and inverse-shifting polymerase chain reaction [PCR]) can be used for detection of the recurrent *F8* intron 22 inversion.<sup>35,46-55</sup> The recurrent *F8* intron 1 inversion can be detected by double PCR<sup>56</sup> or by inverse-shifting PCR.<sup>50</sup> The approach and use of a specific technique depend on the available technical expertise and resources. All results should be confirmed by repeat analytical testing of the DNA sample.
- Depending on the availability of resources, full *F8* or *F9* gene screening is performed by PCR and Sanger sequencing, or next-generation sequencing (NGS), for the detection of missense, nonsense, splice-site, small and large deletions, duplications, and insertions.<sup>46,57-61</sup> Where resources are limited, laboratories may choose a cost-effective screening approach prior to Sanger sequencing,<sup>62</sup> e.g., by heteroduplex analysis using conformation sensitive gel electrophoresis (CSGE).
- When choosing an analytical technique, laboratories must be aware of the sensitivity and specificity of the approach used and the turn-around time for producing an interpretive report. All results should be confirmed by repeat analytical testing of the DNA sample.
- The presence of a variant should be confirmed in both 5' (forward) and 3' (reverse) directions, specifically in heterozygous carriers, when analyzing variants detected using Sanger sequencing.
- In case of no amplification in a particular exon or in a contiguous stretch during PCR, a large DNA deletion may be suspected. This should be confirmed by standard approaches such as gap-PCR or techniques which can detect gene dosage or CNVs such as multiplex ligation-dependent probe amplification (MLPA) or quantitative real-time PCR on the deleted region.<sup>63-71</sup> The conventional Sanger sequencing techniques are not sensitive to pick up CNVs in the case of carriers.
- When a disease-causing variant is not detected, large duplications or insertions may be suspected. These can be

detected by applying the same methods as those employed for identifying large deletions, as described above.

- The technical approach for CNV analysis may depend on the resources available to the laboratory. According to the practical limitations of the technique, results should be provided with an estimation of error, if applicable.
- High-throughput sequencing techniques, e.g., NGS, should only be used after it is established that structural variants can be detected by the technique.<sup>72</sup>
- All results of genetic testing should be confirmed by independent testing of the DNA sample. This may be accomplished either through a repeat of the original assay or by using a different methodology, e.g., using Sanger sequencing to confirm an NGS result.
- During the technical process of taking a sample for prenatal diagnosis, the fetal sample may get contaminated with maternal blood which can lead to misdiagnosis. Different techniques can be used for maternal cell contamination testing depending on the available technical expertise and resources. For example, multiple autosomal short tandem repeat (STR) markers may be used.<sup>73-76</sup> When choosing an analytical technique, laboratories must be aware of the sensitivity and specificity of the approach used and the turn-around time for producing an interpretive report.

##### RECOMMENDATION 4.4.1:

- **For people with severe hemophilia A, or moderate hemophilia A with lower-borderline factor activity levels (FVIII:C 1-3 IU/dL), the WFH recommends testing for the *F8* intron 22 inversion and *F8* intron 1 inversion in the first line of genetic testing.**
- **REMARK: Different techniques can be used for detection of the *F8* intron 22 inversion and intron 1 inversion depending on the available technical expertise and resources.**
- **REMARK: All results should be confirmed by independent analytical testing of the DNA sample. **

##### RECOMMENDATION 4.4.2:

- **For people with severe hemophilia A who are negative for the common *F8* intron 22 inversion and *F8* intron 1 inversion variants, the WFH recommends full gene screening of the essential regions of *F8*, including the 26 exons, splice boundaries, promoter, and 5' and 3' untranslated regions.**
- **REMARK: For example, depending on the availability of resources, full *F8* gene screening may take the form of polymerase chain reaction (PCR) and Sanger sequencing or next generation sequencing (NGS). Where resources**

are limited, laboratories may choose a cost-effective screening approach prior to Sanger sequencing.

- **REMARK:** When choosing an analytical technique, laboratories must be aware of the sensitivity and specificity of the approach used and the turn-around time for producing an interpretive report.
- **REMARK:** The presence of a variant should be confirmed in both 5' (forward) and 3' (reverse) directions, specifically in heterozygous carriers, when analyzing variants detected using Sanger sequencing.
- **REMARK:** All results should be confirmed by independent analytical testing of the DNA sample. **CB**

#### **RECOMMENDATION 4.4.3:**

- For people with hemophilia B, the WFH recommends full gene screening of the essential regions of *F9*, including the 8 exons, splice boundaries, promoter, and 5' and 3' untranslated regions.
- **REMARK:** For example, depending on the availability of resources, full *F9* gene screening may take the form of polymerase chain reaction (PCR) and Sanger sequencing or next generation sequencing (NGS). Where resources are limited, laboratories may choose a cost-effective screening approach prior to Sanger sequencing.
- **REMARK:** When choosing an analytical technique, laboratories must be aware of the sensitivity and specificity of the approach used and the turn-around time for producing an interpretive report.
- **REMARK:** The presence of a variant should be confirmed in both 5' (forward) and 3' (reverse) directions, specifically in heterozygous carriers, when analyzing variants detected using Sanger sequencing.
- **REMARK:** All results should be confirmed by independent analytical testing of the DNA sample. **CB**

#### **RECOMMENDATION 4.4.4:**

- For people with hemophilia A or B in whom no variant is detectable on inversion analysis or full gene sequencing, the WFH recommends that a large deletion or duplication event be investigated.
- **REMARK:** Copy number variation (CNV) analysis may be performed using various validated techniques dependent on the resources available to the laboratory. According to the practical limitations of the technique, results should be provided with an estimation of error, if applicable.
- **REMARK:** All results should be confirmed by independent analytical testing of the DNA sample. **CB**

#### **RECOMMENDATION 4.4.5:**

- For prenatal testing, the WFH recommends maternal cell contamination testing of the fetal sample.
- **REMARK:** Different techniques can be used for maternal cell contamination testing depending on the available technical expertise and resources. For example, multiple autosomal short tandem repeat (STR) markers may be used.
- **REMARK:** When choosing an analytical technique, laboratories must be aware of the sensitivity and specificity of the approach used and the turn-around time for producing an interpretive report. **CB**

## **4.5 | Classification and description of variants**

- The American College of Medical Genetics and Genomics (ACMG) guidelines were developed to provide a standardized approach and terminology for classification of genetic variants in Mendelian disorders.<sup>77</sup> When applied across laboratories, they provide clinicians with useful information on the likelihood that the variant impacts gene function.<sup>6</sup>
- Genetic diagnostics are critically dependent on accurate and standardized descriptions and sharing of genetic variants. The Human Genome Variation Society (HGVS) maintains a sequence variant nomenclature system for this purpose (<http://www.HGVS.org/varnomen>).<sup>78</sup> Providing corresponding *F8* or *F9* legacy nomenclature can be helpful to the clinician for comparison to prior patient or family clinical reports.

#### **RECOMMENDATION 4.5.1:**

- The WFH recommends that variants be classified per the American College of Medical Genetics and Genomics (ACMG) guidelines.
- **REMARK:** ClinGen, a U.S. National Institutes of Health-funded resource dedicated to building a central resource that defines the clinical relevance of genes and variants, has assembled an international expert committee to apply ACMG recommendations to *F8* and *F9* variants, which should produce more hemophilia-specific recommendations. **CB**

#### **RECOMMENDATION 4.5.2:**

- The WFH recommends that variants be described using the Human Genome Variation Society (HGVS) nomenclature. **CB**

## 4.6 | Interpretive reports

- Clinical laboratory reports should include information to allow correct identification of the patient and specimen, report the variant using standardized nomenclature with a genome reference, note limitations of the assay, and provide an interpretation of the findings in a manner that will be helpful to the ordering clinician.<sup>6,79,80</sup>

### RECOMMENDATION 4.6.1:

- The WFH recommends that interpretive reports contain:
  - patient information including patient name, date of birth, ordering clinician, date of specimen collection, diagnosis, baseline factor level, and family pedigree;
  - description of the assay(s), references to the literature (if applicable), limitations of the test, and the genome reference sequence used for analysis;
  - results including DNA variant(s) in Human Genome Variation Society (HGVS) nomenclature and American College of Medical Genetic and Genomics (ACMG) variant classification; and
  - interpretation of test results in a format useful to the ordering clinician, including recommendations for follow-up testing if indicated, implications of test results for patients and family members, and the role of genetic counselling. **CE**

### RECOMMENDATION 4.6.2:

- For all interpretive reports for all individuals undergoing genetic testing for hemophilia, the WFH recommends that the ordering clinician and reporting scientist be available to discuss the potential phenotypic consequences of the reported genotype, as required. **CE**

## 4.7 | Strategies if causative variant is not detected

- Approximately 0.6% of patients with severe hemophilia A and 2.9% of patients with moderate or mild hemophilia A will have no identifiable genetic variant in *F8* genomic DNA using current diagnostic methods, i.e., covering all coding and regulatory regions of *F8* but not deep intronic sequences.<sup>67</sup>
- Approximately 1.1% of patients with moderate or mild hemophilia B will have no identifiable genetic variant in

*F9* genomic DNA using current diagnostic methods that exclude the screening of deep intronic sequences.<sup>67</sup>

- In patients with a clear diagnosis of hemophilia A and no pathogenic variant identified in the *F8* coding sequences, analysis of intronic regions by sequencing or targeted massively parallel sequencing (MPS) to the whole *F8* is an option to detect and analyze deep intronic variants involved in splicing defects, which are suspected to account for most of these patients' phenotypes.<sup>81-86</sup> Deep intronic variants should be interpreted with caution, and functional analysis of these variants would be desirable to demonstrate their pathogenicity.
- NGS platforms have been designed to cover different needs. Among them, the My Life, Our Future platform (<https://www.mylifeourfuture.org>) simultaneously analyzes all small variants and the prevalent inversions causing hemophilia A and B<sup>72</sup>; the ThromboGenomics platform (<http://thrombo.cambridgednadiagnosis.org.uk>) analyzes 63 genes associated with thrombotic, coagulation, and platelet disorders<sup>87</sup>; and the 23-gene NGS panel for inherited bleeding coagulation disorders analyzes 23 genes known to be associated with inherited bleeding disorders.<sup>88</sup> The latter two approaches complement the variant screening with a separate testing of *F8* inversions. Due to the wide range of genes under analysis, the latter two platforms are particularly useful to investigate the hidden cause of bleeding in a patient lacking a proper diagnosis.
- Whole-genome sequencing (WGS) can be considered noting any limitations in detecting structural variation. Linkage analysis may be considered for family studies.<sup>89</sup>
- Complex genomic rearrangements may be considered in some individuals who present with an atypical phenotype. These patients, in whom a large genomic deletion including part or all of *F8* or *F9* is suspected, should be referred to a geneticist to evaluate the possible utility of a pangenomic study. The presence of a contiguous gene syndrome can be analyzed by cytogenetic microarray analysis.<sup>90-93</sup>
- In patients with a confirmed diagnosis of hemophilia A and no *F8* exonic or intronic pathogenic variant detected, identification of specific micro-RNA expression imbalances, either by ncRNA microarrays or RNA-seq (MPS-based transcriptome), may represent the cause for *F8* downregulation and hemophilia A expression.<sup>94-96</sup> However, further research is still necessary to determine the actual role of microRNAs in the pathogenesis of hemophilia A.
- Germline and somatic mosaicism may complicate any genetic assessment in hemophilia.<sup>97,98</sup>

- In some cases, when testing for the familial variant in the mother of a patient with hemophilia, the variant will not be detected. In this instance, the possibility of mosaicism should be considered.
- In hemophilia A-affected probands where the mode of inheritance is not conclusive, or in low-level female probands, other potential diagnoses that need to be investigated include:
  - type 2N VWD if only low FVIII:C level on the phenotypic screen has been assessed;
  - combined FV and FVIII deficiency caused by pathogenic variants affecting *LMAN1* or *MCFD2* genes<sup>99</sup>;
  - other types of VWD.<sup>100</sup>
- See Chapter 3: Laboratory Diagnosis and Monitoring.
- As X-chromosome-linked recessive disorders, hemophilia A and B affect hemizygous males while heterozygous females (carriers) do not typically express hemophilia symptoms. However, in cases of symptomatic carriers, abundant evidence has indicated that non-random and extremely skewed X-chromosome inactivation plays central roles in hemophilia pathogenesis.<sup>11,101</sup> Furthermore, hemophilia expression in female heterozygous carriers is caused by the phase of the X-chromosome inactivation skewing, preferentially silencing the normal *F8* allele.<sup>12</sup>

#### **RECOMMENDATION 4.7.1:**

- **For people in whom a strong diagnosis of hemophilia is certain but no *F8* or *F9* variant is detected using current diagnostic genetic testing, the WFH recommends that other genetic causes be considered (e.g., deep intronic variants).**
- **REMARK: Current testing techniques are expected to evolve in the near future to include next generation sequencing (NGS) and whole genome sequencing (WGS).**
- **REMARK: NGS and WGS techniques should only be used after it is established that structural variants can be detected by the technique. **CB****

#### **RECOMMENDATION 4.7.2:**

- **For “at-risk” female relatives of people with hemophilia in whom the familial variant is not detected using standard diagnostic genetic testing, particularly in females with one affected child, the WFH recommends that the possibility of mosaicism be considered and discussed during genetic counselling. **CB****

#### **RECOMMENDATION 4.7.3:**

- **For people with hemophilia A in whom the mode of inheritance is not conclusive, and in whom no inversion or variant is detected by current diagnostic testing, the WFH recommends that other potential diagnoses be investigated, including type 2N von Willebrand disease (VWD), combined FV and FVIII deficiency, or other types of VWD. **CB****

#### **RECOMMENDATION 4.7.4:**

- **For symptomatic females with low phenotypic coagulation FVIII or FIX levels in whom just one pathogenic variant is found, the WFH recommends performing investigative tests for an X-chromosome inactivation pattern, if locally available. **CB****

## **4.8 | Quality assurance**

- Quality assurance (QA), as described in Chapter 3: Laboratory Diagnosis and Monitoring – Quality assurance, is an umbrella term used to describe all measures taken to ensure the reliability of laboratory testing and reporting. In genetic testing, this covers all aspects of the diagnostic process from nucleic acid extraction and genetic analysis, to the description and classification of the variant(s) detected, and the production of an interpretive report to the ordering clinician.
- Internal Quality Control (IQC) of genetic tests should routinely be performed to ensure the validity of any variant(s) detected.
- Genetics laboratories are strongly advised to participate in External Quality Assessment Schemes (EQAS) to ensure that the quality of their results identified, classified, and interpreted, are in agreement with those obtained by other laboratories. This may be by a formal EQAS or an informal sample exchange between laboratories. Formal EQAS for genomics are provided by, for example, Genomics Quality Assessment (GenQA), and specifically for hemophilia genetic assessment by the U.K. National External Quality Assessment Service (UK NEQAS) for Blood Coagulation.
- Genetic diagnostic laboratories should undergo periodic accreditation, if available, by an approved body. Accreditation assesses the laboratory against internationally agreed standards to ensure high-quality provision of the genetic diagnostic service.
- The formation of Genetics Laboratory Networks for those providing genetic assessment of hemophilia, either within



countries or between those in regions of the world, enables an opportunity for sharing of good practice and expertise.

#### RECOMMENDATION 4.8.1:

- **The WFH recommends that genetic diagnostic laboratories should undergo periodic accreditation, if available, by an approved body.** **CB**

#### RECOMMENDATION 4.8.2:

- **The WFH recommends that internal quality control (IQC) of genetic tests be performed and recorded routinely within the laboratory.** **CB**

#### RECOMMENDATION 4.8.3:

- **The WFH recommends that laboratories participate in external quality assessment schemes (EQAS) for the genetic tests they provide.**
- **REMARK: Participation in an EQAS ensures the provision of a test that is robust and reliable. This may be through participation in a formal EQAS or an informal sample exchange between laboratories.** **CB**

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.