1 Haemophilic syndromes: definition, diagnosis, and update on treatment

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INTRODUCTION

Haemophilia is a rare inherited bleeding disorder caused by the deficiency or functional defect of either clotting factor VIII (FVIII) in Haemophilia A (HA) or IX (FIX) in Haemophilia B (HB). HA is more common than HB, with a prevalence of one in 5,000-10,000 male live births compared to one in 30,000, respectively, across all ethnic and racial groups. HA accounts for approximately 80% to 85% of all cases. Both are transmitted genetically as X-linked recessive disorders.[1]

F8 AND *F9* GENES

The human *F8* gene comprises 186,000 base pairs and is considerably larger than the *F9* gene, which consists of 34,000 base pairs. The *F8* gene maps on the long arm of the X chromosome, in the most distal band Xq28; the *F9* gene is located on the tip of the long arm of the X chromosome at Xq27.1, close to *F8* gene.[2] Considering its size alone, *F8* gene is more susceptible to mutations; this may be an explanation for the greater prevalence of HA versus HB (4:1). *F8* gene contains 26 exons and 25 introns, the spliced FVIII mRNA is approximately 9kb in length and predicts a precursor protein of 2,351 amino acids. After removal of the peptide secretory leader sequence, mature FVIII comprises 2,332 amino acids with the domain structure A1-a1-A2-a2-B-a3-A3C1-C2.[3] FVIII synthesis occurs in the liver by sinusoidal endothelial cells.[4] FVIII circulates in plasma complexed non covalently with von Willebrand factor (vWF), which acts as a plasma carrier, protecting it from proteolysis and rapid clearance. After activation by thrombin (Factor IIa), FVIII dissociates from the complex and interacts with FIXa in the coagulation cascade. FVIII assumes the role of cofactor of FIXa in the activation of factor X, which in turn, with its cofactor factor Va, activates more thrombin. Thrombin splits fibrinogen into fibrin which polymerizes to cross-link (with factor XIII) in a blood clot.[5] *F8* gene defects associated with HA may be divided classically into several categories: gross gene rearrangements, insertions or deletions ranging from one base pair up to the entire gene, and single DNA base substitutions resulting in either amino acid replacement ("missense"), premature peptide chain termination ("nonsense" or stop mutations) or mRNA splicing defects.[6] The most common defect in *F8* gene, responsible for at least 45% of severe HA, involves the inversion of intron 22. This results from

the translocation and exchange of DNA between either of two nonfunctional FVIII-related genes with intron 22 and areas of homologous DNA within the functional *F8* gene.[7] The recombination produces disjointed and inverted DNA sequences, preventing the transcription of a normal full-length FVIII molecule.

F9 gene contains 8 exons and 7 introns, the spliced FIX mRNA is about 2.8 kb in length and predicts a single chain glycoprotein of 415 amino acids. FIX is a vitamin-K-dependent coagulation factor; prior to be secreted from hepatocytes, FIX undergoes gamma–carboxylation, O- and N-linked glycosylation, phosphorylation, sulfation, disulfide bond formation, and beta–hydroxylation, as well as cleavage of the signal peptide to propeptide. FIX plays a critical role in blood coagulation, when activated by FXI or FVII, FIX actives FX in presence of Ca^{2+}, membrane phospholipids, and FVIII cofactor.[8] Numerous pathogenic point mutations and small insertions or deletions have been identified in *F9* gene of HB patients.[9] These frequently result in the production of a defective, nonfunctioning, but immunologically detectable FIX protein in the plasma. Gross genetic abnormalities as gene rearrangements or deletions affecting the whole, or a large part, of the gene are much rarer than HA, and are found only in 7% of HB cases.

SEVERITY OF HAEMOPHILIA

The disease severity in haemophilia is classified according to the plasma level of FVIII or FIX activity, which is determined by the type of the causative mutation in *F8* and *F9*, respectively. It is defined by the measured level of clotting factor activity, assayed in comparison to a reference standard that is assumed to have FVIII/FIX levels of 100%, corresponding to a FVIII/FIX activity of 1.0 U/mL. The FVIII/FIX level in normal population ranges from 50 to 150% (0.50-1.5 U/mL). Subjects with factor plasma levels <0.01 IU/mL are classified as severe haemophiliacs, whereas those with factor levels between 0.01 and 0.05 IU/mL and >0.05 to 0.4 IU/mL have moderate and mild haemophilia.[10] Although the bleeding phenotype may be rather heterogeneous,[11] this classification reflects closely the severity and frequency of clinical symptoms (**Table 1.I**). Patients with severe haemophilia frequently develop haemorrhages into joints, muscles or soft tissues without any apparent cause. They can also suffer from life-threatening bleeding episodes such as intracranial

Table 1.I. Clinical classification of Haemophilia A and B.

Classification	FVIII or FIX activity	Clinical manifestations
Severe	<1% of normal (0.01 IU/mL)	Spontaneous haemorrhage from early infancy Frequent spontaneous hemarthroses and haemorrhages, requiring clotting factor replacement
Moderate	1-5% of normal (0.01-0.05 IU/mL)	Haemorrhage secondary to trauma or surgery Occasional spontaneous hemarthroses
Mild	>5-40% of normal (0.0-0.40 IU/mL)	Haemorrhage secondary to trauma or surgery Rare spontaneous hemarthroses

haemorrhages. Persons with mild and moderate factor deficiency rarely experience spontaneous haemorrhages, and excessive bleeding mostly occurs only following trauma or in association with invasive procedures.

CLINICAL MANIFESTATIONS

Traditionally HA and HB have been considered clinically indistinguishable, with musculoskeletal bleedings, particularly in joints, as hallmark of a severe haemophilia. Some evidence, however, suggest that patients with severe HB may have a less severe bleeding phenotype, a lower bleeding frequency and better long-term outcomes, compared to severe HA patients.[12, 13]

Natural history of severe haemophilia showed that about 50% of children suffer from the first joint bleeding within the first year of life,[14] and 90% experience it before the age of 4.5 years.[15] Recurrent joint bleeding result in chronic, crippling haemophilic arthropathy. The most involved joints are knees, elbows and ankles.[16] The first sign of a joint bleeding is a sensation of intraarticular burning, followed by fullness, tightness, swelling, and increasing pain leading to limitation of motion. Involuntary muscle splinting due to pain induces joint immobilization and initiates a vicious cycle of atrophy and contracture. Intrarticular deposition of haemosiderin from red blood cell lysis contributes to the synovial inflammation and increased vascularity, predisposing to further bleedings. Joints with a chronically inflamed and hypertrophic synovium are referred to as "target joints" and are susceptible to recurrent bleedings unless treated.[17] Deep muscle is the second most common site of bleeding in HA and HB. The site of bleeding determines the morbidity of the event. Bleeding into large muscles, though extensive, generally resolve without complications, as they are not confined. Bleedings into a closed fascial compartment can lead to compression of vital structures with resulting in a compartment syndrome characterized by ischemia, gangrene, flexion contractures, and neuropathy. Bleeding into the ilio-psoas muscles and retroperitoneal space can produce sudden onset of inguinal pain and decreased range of motion of the ipsilateral hip, which assumes a flexed position, usually with external lateral rotation. Bleeding can be life-threatening when a large volume of blood in the peritoneum is lost. In addition, femoral nerve compression can occur with permanent disability if a compartment syndrome develops. A clinical manifestation found in 1-2% of patients with severe haemophilia is pseudotumor, a cystic lesion in the subperiosteal area of bone or in soft tissues, outcome of recurrent bleeding with enlargement and encapsulation. Pseudotumors are composed of old clots and necrotic tissues, arising after inadequate treatments. Symptoms associated with expanding pseudotumors are related to the size of the mass and the degree of compromise of the integrity of the structure they are invading. Spontaneous gross hematuria can occur frequently in PWH and is usually painless unless intraureteral clots develop. Its cause is often unknown with undetectable structural lesion. Hematuria is sometimes self-limited to few days, but it may persist for weeks or months if untreated. Gastrointestinal bleeding occurs in 10-15% of adult PWH. Spontaneous gastrointestinal haemorrhages are rarer than those caused by anatomic lesions; in PWH with cirrhosis HCV related to previous use of infected clotting factor concentrates, varices resulting from portal hypertension are the leading cause of acute bleeds. Intracranial haemorrhage (ICH) is the most common cause of death from bleedings in HA and HB and it can occur after a minimal trauma, particularly in children, or

spontaneously. ICH in children is more frequent under the age of 2 years and in those who have severe haemophilia and are not yet treated with prophylaxis. ICH in adults is often related to arterial hypertension and to the presence of inhibitors against FVIII/FIX.[18] Fifty percent of patients with ICH have neurological sequelae, and 30% events results in death. Today, thanks to the improved understanding of the disorder and development of efficacious therapy based on prophylactic treatment, PWH have a virtually normal life expectancy and quality of life.[19] With the aging of PWH, currently cardiovascular disease represents an emerging medical issue, with a prevalence of 15%[20] and not negligible management difficulties.

DIAGNOSIS

About two thirds of people with haemophilia (PWH) have a family history of the disease, while in the remaining 30% haemophilia is due to a de novo mutation. When family history is present and familial pathogenetic mutations are known, prenatal diagnosis of haemophilia may be made with amniocentesis or chorionic villus sampling. In children at risk of inheriting haemophilia-causing mutations, testing can be done at birth to look for low clotting factor levels. This is often done on a sample of blood from the umbilical cord.

Instead, the post-natal diagnosis of haemophilia is based in the first instance on family and personal history, from which emerge past spontaneous and/or post-traumatic bleeding, especially in the severe form of disease. In mild haemophilia the bleeding history can be negative. HA and HB are characterized by prolonged activated partial thromboplastin time (aPTT) and normal prothrombin time (PT). The prolonged aPTT, due to a deficiency disorder, is fully corrected by performing the mixing test, in which the patient's plasma is mixed 1:1 with normal pooled plasma.

HA and HB diagnosis are made by performing the specific assay of FVIII or FIX clotting activity (FVIII:C or FIX:C) in patient's plasma. FVIII:C and FIX:C measurement can be performed by two methods:

1. the One-Stage Assay (OSA) measures the ability of a plasma containing the factor under investigation to correct or shorten the aPTT of a FVIII or FIX- deficient plasma, after activation of the contact phase and recalcification;
2. the Chromogenic Assay (CSA), in which the sample test is added to a mixture containing FIXa, calcium, and phospholipids so that the only limiting factor is the amount of FVIII or FIX in the sample. The FXa formed in the mixture is detected using a substrate containing a chromophore group whose hydrolysis forms the para-nitroaniline measured by absorbance at 405 nm by a spectrophotometer.

The results of both assays are expressed as % of normal plasma activity or as IU/dl. The laboratory diagnosis and monitoring of FVIII and FIX have used OSA for many years. CSA have been available more recently and has the advantage that it is not influenced by the presence of heparin or antiphospholipid antibodies.[21] Discrepancies in results between the OSA and CSA have been described in approximately 30% of patients with mild HA. In certain discrepant groups with restricted *F8* gene mutations, the OSA results are more than 1.5-fold higher than CSA, with the inherent risk of missing or misleading correct diagnosis. WFH

recommends the use of CSA for the diagnosis of mild HA.[22,23] Differences between OSA and CSA have been also reported in the measurement of some standard (SHL) and extended half-life (EHL) factor concentrates and gene therapy using different aPTT reagents.[24] Finally, the measurement of bispecific antibody therapy in PWHA has highlighted differences between chromogenic assays.

THERAPEUTIC MODALITIES FOR HA AND HB

Haemophilia treatment centers (HTCs) provide comprehensive medical and psychosocial services to patients with inherited bleeding disorders. HTCs, consisting of multidisciplinary teams, offer to PWH and its complications the more appropriate and innovative treatments. In Italy only HTCs recognized at a regional level can prescribe replacement products, based on individual therapeutic plans.

CLOTTING FACTOR REPLACEMENT THERAPY

The mainstay of haemophilia treatment is replacement of missing clotting factor administered either on demand or in prophylaxis.[1] Concentrates of FVIII and FIX are widely available, both plasma-derived (pd-) or produced with recombinant (r-) DNA technology, subjected to multiple procedures of inactivation/viral exclusion, which offer advantages of handling and safety guarantees. Pd- FVIII and pd-FIX concentrates are currently obtained from Penta-NAT controlled plasma (HCV-RNA, HIV-RNA, HAV-RNA, HBV-DNA and Parvo-B19 DNA). All concentrates are then subjected to different viral inactivation techniques as dry heating, pasteurisation, vapour and solvent/detergent procedures, ultrafiltration and nanofiltration steps. Viral safety is mandatory because until the 1985, non-virally inactivated products from single donor and pd-concentrates obtained from pools of donors transmitted hepatitis viruses B and C (HBV, HCV) to more than 90% of patients treated with replacement therapy worldwide.[25] The epidemic spread of human immunodeficiency virus (HIV) infection affected about 30% and 70% of HA and HB patients respectively.[26]

Factor replacement products are classified based on their final purity, defined as specific activity (International Units of clotting factor activity/mg of protein). Intermediate purity products have relatively low specific activities (less than 50 U/mg) because they are contaminated with additional plasma proteins; high-purity (more than 50 U/mg) and ultra-high-purity (more than 3000 U/mg for FVIII concentrates; more than 160 U/mg for FIX concentrates) products contain little or no contaminating plasma proteins other than albumin as a stabilizer. The first-generation r-FVIII proteins were stabilized with bovine or human serum albumin (HSA) either in preparation or in final formulation. Thus, in second-generation therapies HSA in the final formulation has been replaced by non-protein stabilizers and third-generation products lack added bovine and/or human protein in either the cell culture procedure or in the final formulation. Albumin-free formulations of recombinant "full-length" and B-domain deleted or truncated FVIII concentrates are available, as a single-chain r-FVIII molecule, with a truncated B-domain and the heavy and light chains covalently linked to form a stable and homogenous drug that binds with high affinity to VWF. Monoclonal antibody-purified, pd-IX concentrates and r-FIX concentrates are free of albumin.

Table 1.II. Available standard and extended half life rFVIII concentrates with with recommended schemes for prophylaxis.

SHL-FVIII concentrates Product	Characteristic	Prophylaxis scheme according to data sheet
Octocog alfa (Recombinate®)	First generation full lenght rFVIII	20-40 IU/kg at 2–3-day intervals.
Octocog alfa (Advate®)	Third generation full lenght rFVIII	20-40 IU/kg at 2–3-day intervals. <6 years: 20-50 IU/kg 3-4 days per week.
Moroctocog alfa (Refacto AF®)	Second generation B-domain deleted rFVIII	20-40 IU/kg at 2-3 days intervals.
Octocog alfa (Kovaltry®)	Full lenght rFVIII with superior glycosylation	20-40 IU/kg 2-3 times a week. <6 years: 20-50 IU/kg 2-3 days per week or every other day.
Turoctocog alfa (Novoeight®)	B-domain truncated rFVIII fully sulphated in Tyr1680	20-40 IU/kg every 2 days or 20-50 IU/kg 3 days a week. Adults and adolescents also: 40-60 IU/kg every 3 days or two days a week.
Simoctocog alfa (Nuwiq®)	B-domain deleted rFVIII fully sulphated and preserved N-glycosylation. Only rFVIII produced by human cell line	20-40 IU/kg at 2–3-day intervals.
Lonoctocog alfa (Afstyla®)	Single chain rFVIII with stronger affinity to VWF	20-50 IU/kg 2-3 days per week. Pediatric patients: 30-50 IU/kg 2-3 days per week.
EHL-FVIII concentrates		
Efmoroctocog alfa (Elocta®)	Fusion protein with the Fc fragment of IgG1 (rFVIIIFc)	50 IU/kg at 3-5-day intervals. Dose can be adjusted in a range between 25 and 65 IU/kg. Children under the age of 12 may need higher or more frequent doses.
Rurioctocog alfa pegol (Adynovi®)	Random PEGylation of rFVIII	40-50 IU/kg twice a week with intervals of 3-4 days. Adjustments of doses and intervals between doses can be considered based on FVIII levels achieved and individual bleeding phenotype.

Damoctocog alfa pegol (Jivi®)	45-60 IU/kg every 5 days. Depending on the clinical characteristics of the patient, this dose may also be 60 IU/kg every 7 days or 30-40 IU/kg twice a week	45-60 IU/kg every 5 days. Depending on the clinical characteristics of the patient, this dose may also be 60 IU/kg every 7 days or 30-40 IU/kg twice a week. In overweight patients, the maximum prophylactic dose per infusion should not exceed 6,000 IU.
Turoctocog alfa pegol (Esperoct®)	Single site-specific PEGylation of rFVIII	50 IU per kg of body weight every 4 days. Dose adjustments and dose intervals can be considered based on factor VIII levels and individual bleeding tendency.

The dosing of clotting factor replacement therapy is based on several factors: age, patient's plasma volume; distribution of the clotting protein between the intravascular and extravascular compartments; circulating half-life of the clotting factor in the plasma; and level of activity required to achieve adequate haemostasis or prophylaxis. Dosages are calculated by assuming that 1 U/kg body weight of FVIII replacement will raise the plasma activity of FVIII by approximately 0.02 U/mL (2%), and 1 U/kg of FIX concentrate, which has a larger volume of distribution, will increase plasma FIX levels by 0.01 U/mL (1%). The circulating half-life for endogenous FVIII is 8 to 12 hours, and for endogenous FIX is around 18 hours. Available r-FVIII and r-FIX concentrates can be SHL or EHL. Different technologies are applied to extend half-life: the conjugation with polyethylene glycol and the production by genetic engineering of fusion proteins containing the FVIII and FIX linked to a long-lived plasma protein such as albumin or the Fc fragment of immunoglobulin (Ig)G, available for FIX only (**Tables 1.II, 1.III**). The prolongation of half-life for r-FIX is relevant, with half-life extension to up to 100 hours, allowing substitution intervals of 1-2 weeks. Due to its greater size and the ceiling influence of endogenous VWF, which is the carrier of FVIII, the effect for r-FVIII products so far is only moderate, as the half-life extension is limited to about 15-18 hours (around 1.5-fold rFVIII).

Efanesoctocog alfa is the first high-sustained FVIII replacement product with pharmacokinetic parameters that are independent of VWF.[27] Several modifications have been made to extend its plasma half-life.[28] To decouple it from VWF and overcome the VWF-imposed half-life ceiling, a recombinant D'D3 domain of VWF was appended to efanesoctocog alfa. This stabilizes the molecule in plasma and prevents its association with endogenous VWF. Additionally, efanesoctocog alfa features a dimeric Fc domain (for recovery from the lysosomal degradation pathway) and two XTEN polypeptide domains (which reduce renal clearance and shield the protein from proteolytic degradation.[29] This new FVIII concentrate provides three- to four-fold longer haemostatic control compared with available SHL and EHL-FVIII replacement products on the market,[28] allowing high trough levels (between 10 and 40 IU/dL) of FVIII to be maintained with once-weekly administration.[30] Efanesoctocog alfa is not yet prescribed but is in an advanced phase of clinical trial.

Table 1.III. Available standard and extended half-life rFIX concentrates with with recommended schemes for prophylaxis.

SHL-FIX concentrates		
Product	**Characteristic**	**Prophylaxis scheme according to data sheet**
Nonacog alfa (Benefix®)	Third generation r-FIX	40 IU/kg every 3-4 days. In some cases, especially in younger patients, shorter or higher dose intervals may be required.
Nonacog gamma (Rixubis®)	Third generation r-FIX	Patients >12 years: 40-60 IU/kg every 3-4 days. Patients <12 years: 40-80 IU/kg every 3-4 days.
EHL-FIX concentrates		
Albutrepenonacog alfa (Idelvion®)	Fusion protein with albumin fusion (rFIX-FP)	Patients >12 years: 35-50 IU/kg once a week. Some patients who show optimal control of the disease with a regimen of administration once a week may pass, with a dose of up to 75 IU/kg, at intervals of administration every 10 or 14 days. Patients <12 years: 35-50 IU/kg once a week. For patients >18 years of age, further extension of the range of administration may be considered.
Eftrenonacog alfa (Alprolix®)	Fusion protein with the Fc fragment of IgG1 (rFIXFc)	Patients >12 years: 50 IU/kg once a week, adjusting the dose according to individual response, or 100 IU/kg once every 10 days, adjusting the interval according to individual response. Patients <12 years: 50-60 IU/kg every 7 days. Some well-controlled patients with a once-every-10-day regimen may possibly be treated with an interval of 14 days or more.
Nonacog beta pegol (Refixia®)	Site-specific GlycoPEGylation of rFIX	40 IU/kg body weight once a week. Dose and interval adjustments may be considered based on FIX levels and individual bleeding tendency.

Optimal haemostatic plasma levels for FVIII and FIX depends on the different clinical situation. On-demand replacement treatment should be administered at early onset of symptoms to limit the amount of bleeding and to prevent damage. Replacement therapy should also be given before surgery to prevent intraoperative bleeding complications (**Table 1.IV**) or prophylactically to prevent haemophilic arthropathy.[22] According to the WFH guidelines,[22] for patients with a severe phenotype, including those with moderate haemophilia, long term