Hemophilia A: Diagnosis and Management

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4.1 Introduction

The word "**Hemophilia**" (loosely translated to love of bleeding) was first used by Hopff in 1828 [1]. Hemophilia (either A or B) is a bleeding disorder that is inherited in an X-linked recessive manner. The most common type of hemophilia is hemophilia A, caused by a defect or deficiency in coagulation factor (F) VIII [2]. Hemophilia A or classical hemophilia is the most common severe bleeding disorder comprising ~80% of all types of hemophilia [2]. The main feature of hemophilia A is prolonged and abnormal bleeding, especially into joints and soft tissues.

The precise incidence of the disorder is unknown, but the estimated incidence ranges between 5 and 20 per 100,000 males, with the majority of patients remaining undiagnosed [3]. Approximately 40–60% of patients with hemophilia A suffer the severe form of the disease, and they are at risk of bleeding after surgery, such as dental extraction and circumcision, or traumatic visceral hemorrhages. They also exhibit spontaneous bleeding without trauma or injury or other interventional causes. Intra-articular joint hemorrhage is the most common spontaneous bleeding,

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and this can lead to chronic physical disability due to arthropathy. In fact, currently, the majority of severe hemophilia develops chronic disability, typically in the form of hemophilic arthropathy, because of repeated joint bleeds [4].

Hemarthrosis is the main cause of functional disability and even psychological depression of patients with hemophilia [4]. Hemorrhage into the central nervous system (CNS) is the leading cause of death in hemophilia, accounting for ~20% of non-infective deaths, as reported in developed countries [5]. Primary prophylaxis is the treatment of choice in patients with severe hemophilia A. The goal is to maintain FVIII levels at a concentration that avoids most bleeding episodes, especially microscopic joint bleeds. Several therapeutic choices are available including fresh frozen plasma (FFP) and cryoprecipitate as traditional options and plasma-derived FVIII concentrate and recombinant FVIII (rFVIII) and non-factor concentrates as more advanced choices, but the most promising therapeutic strategy is gene therapy [6].

Based on FVIII activity, hemophilia A is classified into mild (5–40%), moderate (1–5%), and severe (<1%) deficiency. FVIII coagulant activity (FVIII:C) can be measured by either one-stage or chromogenic assays, but chromogenic assay is recommended for FVIII:C assay in emicizumab use [7, 8].

4.2 Factor VIII Structure and Function

The mature FVIII protein consists of three homologous A domains, two homologous C domains, and a unique B domain, which are arranged in the order of A1–A2–B–A3–C1–C2 from the N-terminus to the C-terminal end of protein (Fig. 4.1) [9, 10].

The A1 and A2 domains have about 30% homology with each other. A1–A2–B domains compose the heavy chain of FVIII, while the A3–C1–C2 domains comprise the light chain of the FVIII protein. The two chains are bound non-covalently and require a metal ion-dependent linkage, with the responsible residues located within the A1 and A3 domains. Each domain contains a copper atom [11, 12]. Two main antibody epitopes have been detected in the A2 domain. One epitope is located at amino acids 484–508, while the other is placed between amino acids 558 and 565. Immunization against these epitopes affects FVIII's interaction with activated FIX (FIXa) [13, 14]. There are three acidic peptides named a1, a2, and a3, which are clusters of Aspartic and Glutamic residues and surround the A domains.

The C domains of FVIII and FV are structurally related discoid domains and belong to the phospholipid-binding domain family [15]. The B domain is unique, without significant homology with any other known protein [16]. This domain does

Fig. 4.1 Structure of factor VIII protein; Human factor VIII is a single chain protein with a molecular weight of 300 kDa, and consists of six domains including A1–A2–B–A3–C1–C2. The mature factor VIII protein consists of three homologous A domains, two homologous C domains, and a B domain

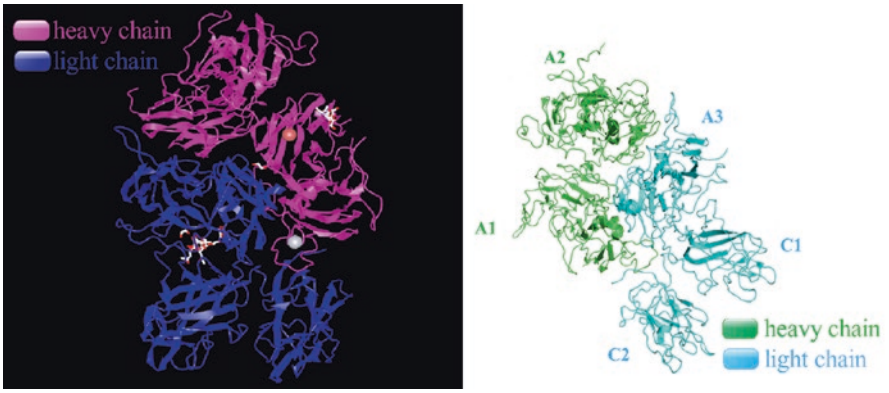

Fig. 4.2 Three-dimensional crystalloid structure of B domain deleted factor VIII protein. There are three A domains: A1, A2, and A3, two C domains: C1 and C2 domains. PDB of factor VIII crystal structure was downloaded from Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (www.pdb.org)

not affect FVIII activity in the process of blood coagulation. When the B domain is cleaved and released, FVIII is converted to the active form (FVIIIa). The protein in active form consists of a heavy chain and a light chain. This process of activation requires two cleavages by thrombin (Fig. 4.2).

The different domains of the FVIII protein have different functions. An acidic peptide, which spans from amino acid 337–374 and called a1, separates the A1 from the A2 domain. Second acidic peptide, a2, links the A2 to the B domain. The next short acidic peptide termed a3 is connected with the A3 domain. A pair of homologous C domains (C1 and C2) resides at the C-terminal of FVIII protein. A region of the C2 domain contains a membrane binding site and the site for interaction with von Willebrand factor (VWF) [17]. The 2303–2332 sequence of the C2 domain has been suggested to form an interactive site for both VWF and phospholipids. The organization of the FVIII domains and linker peptides can thus be ordinated as A1.a1.A2.a2.B.a3.A3.C1.C2. Notably, there are seven disulfide bonds within the FVIII molecule: two in each A domain and one within the C1 domain [18]. In the A1 domain, Cys310, Met320, His267, and His315 create a copper-binding site and Cys2000, His1954, and His2005 constitute another copper-binding site in the A3 domain [12, 19].

The first cleavage of FVIII by thrombin at Arg1689 (at the B-a3) position generates a heavy chain with variable-size (90–210 kDa) and consists of A1 and A2 domains and heterogeneous fragments of the partially proteolyzed B domain; during this process, a 40-amino-acid acidic peptide (a3) is released from the C-terminal product to form a 73 kDa product that includes A3–C1–C2 domains, which is called the light chain. Further cleavage by thrombin cleaves the protein between the A1 and A2 domains and removes most of the B domain: cleavage at Arg372 (between the A1 and A2 domains) and at Arg740 (between the A2 and B domains) generates a 54 kDa A1 and a 44 kDa A2 domain (Fig. 4.3) [20, 21].

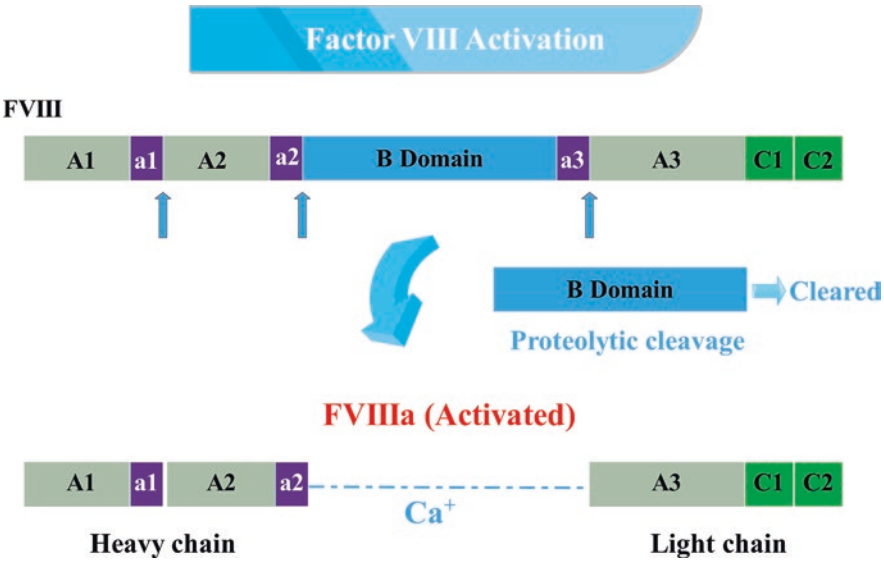

Fig. 4.3 Activation of factor VIII. Cleavage by thrombin removes the B domain and divides factor VIII to two chains: the heavy and light chain. Then the calcium cations link them together. Factor VIII in its nonactivated form circulates in plasma in a complex with von Willebrand factor (vWf). Upon activation by thrombin or factor Xa-mediated site-specific proteolysis, activated FVIII (FVIIIa) serves as a cofactor for factor IXa. Both chains form a non-covalently linked complex in a calcium-dependent manner. This complex is the pro-coagulant activated FVIII (FVIIIa) [22]. Next step in coagulation cascade is activation of FX by tenase complex, which consists of FVIIIa, and FIXa, platelet surface phospholipids and Calcium cation (Table 4.1) [23]

4.3 Hemophilia A

The word hemophilia comes from two Greek words: the "**haima**," which means blood, and the "**philia**" meaning affection. Hemophilia A or classic hemophilia is an X-linked recessive disorder characterized by dysfunctional blood clotting, due to a mutation in the gene for the clotting component, FVIII. This condition was described in the second century AD, when the Talmud, a collection of Jewish Rabbinical writings stated that male newborns should not be circumcised if their two brothers had already died from this procedure. During the twentieth century, hemophilia is estimated to affect one in 5032 live male births in the United States. Approximately 70% of mothers who have a child with hemophilia have a family history. While there is a history of hemophilia, 30% have a negative family history, and this is the first time a hemophilic child is present. These mothers are considered sporadic cases. [24, 25].

Clinical presentations of patients with hemophilia A vary from a mild condition to a severe bleeding tendency. Approximately 50% of people with hemophilia A have the severe form of the disease. Patients with hemophilia A are at higher risk of post-traumatic bleeding such as post-dental hemorrhage and post-surgical bleeding. Spontaneous bleeding is relatively common in patients

Table 4.1 Characteristics of factor VIII domains

Domains	Numbers of amino acids	Function	Molecular manifestation of the defected domain
A	(FVIII A domains consist of: A1(336 aa), A2 (337 aa) and A3 (329 aa))	– Coagulation FVIII A domains are homologous with the ones of coagulation FV and ceruloplasmin. – 40% amino acid identity with each other and to the A domains of FV	–
A1	336	– Creates the heavy chain (A1–A2) – Contains single copper atom	– Reduced stability of FVIIIa – Impaired thrombin activation
A2	337	– Creates the heavy chain (A1–A2) – The main antibody epitope is present in the A2 domain in amino acid 484–508 sites	– Alter FVIII intracellular trafficking and/or secretion – Alter interaction with FIXa – Impaired thrombin activation
A3	329	– Creates the light chain (A3–C1–C2) linked by metal ions – Contain single copper atom	– Alter interaction with FIXa – Reduce stability of the FVIIIa
B[a]	907	– Creates the heavy chain (A1–A2) – Intracellular synthesis quality control: enables interaction of FVIII with chaperone proteins that distinguish properly folded tertiary structure of proteins; stabilizes folded domains, prevents aggregation – Secretion: interacts with cargo-specific sorting receptor complex that enables endoplasmic reticulum to Golgi transport; increases secretion efficiency – Plasma activation: possibly shields thrombin activation site from premature proteolysis – Platelet binding: decreases affinity of inactivated FVIII for activated platelets, thus preserving circulating FVIII – Inactivation: reduces proteolysis by activated protein C and FXa – Clearance: may play a role in FVIII quality control through interaction with asialoglycoprotein receptor	–

continued

Table 4.1 Continued

Domains	Numbers of amino acids	Function	Molecular manifestation of the defected domain
C1	153	– C1 domain an impact on VWF and C2 domain linkage strengthening – Creates the light chain (80 kDa) (A3–C1–C2) linked by metal ions	– Reduction of FVIII binding to VWF – FVIII intracellular trafficking and/or secretion – Misfolded protein – Defect at phospholipid binding surface
C2	160	– C2 domain surface responsible for phospholipid linkage to coagulation FVIII – Creates the light chain (80 kDa) (A3–C1–C2) linked by metal ions	– Reduce the FVIII secretion rate – Misfolded protein – reduction of FVIII binding to VWF – Defect at phospholipid binding surface
a1	37	–	Impaired thrombin activation
a2	31	–	Impaired thrombin activation
a3	42	–	– Impaired thrombin activation – Reduction of FVIII binding to VWF

aa Amino acids, *FVIIIa* activated factor VIII, *FIXa* activated factor IX, *FXa* activated factor X, *VWF* von Willebrand factor
[a]Comprising 40% of FVIII mass

Table 4.2 Classification of hemophilia A and severity of clinical presentations

Severity of disease	Factor level % activity (IU/mL)	Sign and symptoms	Relative incidence	Age at diagnosis
Severe	<1% (<0.01)	Spontaneous bleeding, predominantly in joints, muscles, and internal organs	50%	First year of life
Moderate	1–5% (0.01–0.05)	Occasional spontaneous bleeding, severe bleeding after trauma or surgery	30%	Up to age 5–6 years
Mild	5 – 40% (0.05–0.40)	Severe bleeding with major trauma or surgery	20%	Often later in life

with severe hemophilia A. Recurrent intraarticular joint hemorrhage can lead to chronic disability as a result of arthropathy causing loss of a range of motion and severe pain. In fact, the majority of people with severe hemophilia A develop chronic disability, typically in the form of hemophilic arthropathy, because of repeated joint bleeds [4]. Furthermore, people with severe hemophilia have less than 1% of the normal level of FVIII in their blood. They can experience multiple spontaneous bleeding episodes per month. People with moderate hemophilia usually bleed less than severely affected patients, and post-minor traumatic hemorrhages can occur. Finally, people with mild hemophilia A have even fewer hemorrhages. Sometimes they are not aware of their bleeding problem although an invasive procedure such as surgery or a tooth extraction can bring the issue to light (Table 4.2) [26].

Diagnosis of this disorder can be made based on clinical presentations, appropriate laboratory assessment, and familial history. Because hemophilia A is an X-linked disease, females can be carriers. Carriers have one normal and one abnormal gene. They have a 50% chance of giving the hemophilia gene to each child. Boys who receive the abnormal gene will have hemophilia; girls who inherit it will become carriers. Therefore, if the father of a family has hemophilia and the mother has a hemophilic gene, their daughter can have hemophilia. With regards to phenotype, carriers are usually unaffected or only mildly affected with some bleeding symptoms. Carriers who have >50% of clotting FVIII levels generally do not demonstrate a bleeding tendency [27]. But half of them have less than 50% FVIII and therefore manifest a bleeding tendency proportional to how low the level is in their blood.

Carriers are divided into two groups: (1) mandatory carriers, who necessarily carry the affected X chromosome, and (2) possible carriers [27].

Mandatory Carriers Are

- All female offspring of a man with hemophilia
- Mothers who have a boy with hemophilia and at least one other family member with hemophilia (brother, grandparent, uncle, nephew, or cousin)
- Mothers who have a boy with hemophilia and a family member carrying the hemophilia gene (mother, sister, mother's grandmother, aunt, or uncle)
- Mothers of two or more boys with hemophilia

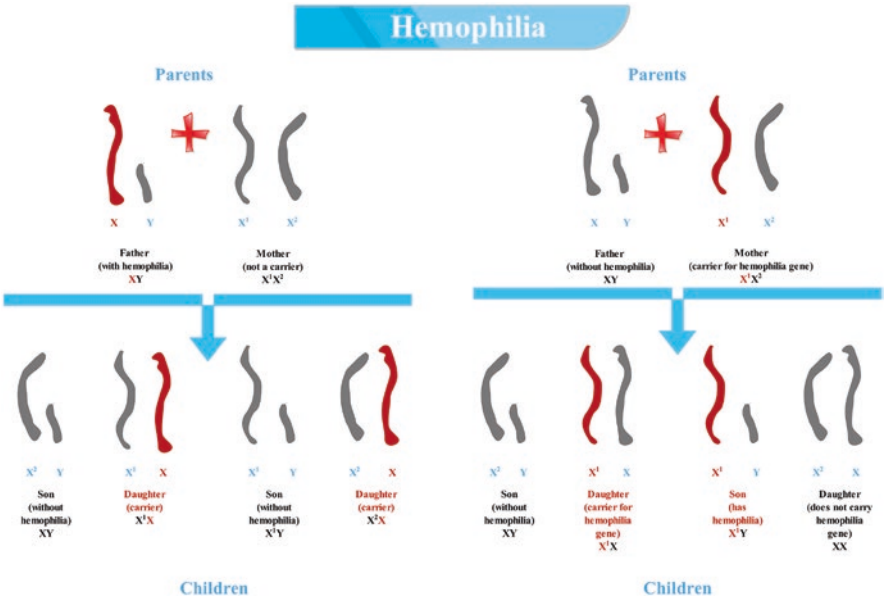

Fig. 4.4 Hereditary chance of giving the hemophilia gene in each child: left, when the father is affected, right, when the mother is a carrier

Possible Carriers Are

- All girls born of a carrier
- Mothers of one boy with hemophilia who have no other family members who have hemophilia or are carriers of hemophilia
- Sisters, mothers, maternal grandmothers, aunts, nieces, and female cousins of carriers (Fig. 4.4)

4.4 Clinical Manifestations

Hemophilia A is the most common severe congenital bleeding disorder, and patients suffer variable clinical presentations. There is a direct correlation between FVIII coagulant activity (FVIII:C) and severity of clinical presentations. Based on residual FVIII:C level, the disorder is classified into severe (<0.01 IU/dL (<1% of normal)), moderate (0.01–0.05 IU/dL (1–5% of normal)), and mild (0.05–0.40% IU/dL (5–40% of normal) deficiency [28].

Mild, moderate, and severe forms comprise 20%, 30%, and 50% of all cases with hemophilia, respectively [29]. Individuals with FVIII:C higher than 30% are usually asymptomatic, but a mild bleeding tendency presents in hemophilia A carrier females with low to low-normal FVIII:C level [30].

Severely affected individuals present with severe bruising and joint hemorrhage during their first 2 years of life, often bleeding with circumcision [31].

Phenotypically, hemarthrosis (particularly in the knee, ankle, and elbow joints), soft-tissue hematoma, easy bruising, retroperitoneal bleeding, intracranial hemorrhage (ICH), and post-surgical bleeding are the most concerning problems in hemophilia A that may present at any age [32]. ICH is the main concern of newborns that can occur immediately after birth or during birth. Severity of the hemophilia and type of delivery can affect the rate of ICH in neonates. Neonates with forceps delivery and Cesarean section delivery are at higher risk of ICH, while non-traumatic normal vaginal delivery imposes less risk. Overlaying the incidence of ICH in hemophilia is variable, but 3.5–4% is estimated to be typical (Table 4.3).

In early childhood, large hematomas might also be observed following intramuscular injections (e.g., immunizations) in boys and should persuade the physician to rule out a bleeding disorder [2, 33].

Bleeding symptoms also may appear later when the teeth erupt. Prolonged severe oral bleeding can lead to anemia [34].

As the child's activity increases with age, the presentation of bleeding episodes is changed. During the toddler age, bumps and falls can cause prolonged oral bleeding from the frenula, superficial hematoma and intramuscular hemorrhages. Physicians must thoroughly examine the patients who have such symptoms to differentiate hemophilia from child abuse. The family history of bleeding disorders can be helpful [2, 35].

Spontaneous hematuria may occur in children and adults, but usually disappears in a few days [36, 37]. Large intramuscular hematoma is also a major bleeding problem in childhood and adulthood periods and should immediately be treated by clotting factor concentrate. Bleeding into a muscle causes it to be swollen hard and tender. Hemorrhages in large muscles may cause anemia and severe pain [2, 38]. Bleeding into iliopsoas may present as an acute abdominal pain and could be mistaken as appendicitis or other intraabdominal surgical emergencies [39]. On the other hand, some mouth problems can occur during infancy, when teeth's roots of infant are mobile. Bleeding also occurs when a tooth falls out or is extracted by a dentist. The teenage years sometimes need for orthodontic braces. The orthodontist must be aware of the child's disorder and should consider the necessary considerations. Another problem is Gum diseases. Chronic periodontitis (pyorrhoea), which is typically seen in adults, and gingivitis are both painless, but may lead to gum bleeding, particularly during brushing [33]. Furthermore, one study suggests that temporomandibular joint open/close movements may result in silent bleeding into the chin joints in patients with inherited coagulation disorder. This study demonstrated that two patients with severe hemophilia developed temporomandibular disorders [41].

Moreover, gastrocnemius, quadriceps and forearm bleedings may increase pressure on neurovascular bundles, causing a situation that is known as compartment syndrome. It can lead to ischemia, fibrosis, neuropathy, atrophy and even may require amputation [42].

Table 4.3 Clinical manifestations of congenital factor VIII deficiency

Prevalence	Bleeding episodes	Iran% (*N*: 885)[a]	North-Eastern Iran% (*N*: 287)	Guidelines for the management of hemophilia (%)	India%)*N*: 56)[b]	Pakistan%)*N*: 229(	Iran% (*n*: 100)	Iran%	
								Severe/ moderate (*n*: 50)	Mild (*n*: 50)
~20–100%	Ecchymosis	13.2	71	–	–	–	–	–	–
	Hemarthrosis	8.7	72.6	70–80	73.21	–	86	86	6
	Oral cavity bleeding	–	–	–	–	–	64	64	30
	Post-partum bleeding	–	–	–	–	–	36	–	–
	Post-operative bleeding	–	–	–	–	18.4	–	76	30
	Post-dental extraction bleeding	6.8	89	–	–	0.9	–	–	–
	Epistaxis	–	55.9	–	26.78		20	20	12
~5–20%	Post-circumcision bleeding	14.5	–	–	–	62	–	–	–
	Gastrointestinal bleeding	0.4	21	–	3.57	–	10	10	0
	Hematuria	–	32.3	–	1.78	–	12	12	0
	Hematoma	1.2	4.2	–	–	0.9	93	82	8
	Skin bleeding	–	–	–	80.35	–	–	–	–
	Muscle bleeding			10–20	46.42	–	–	–	–
~<5%	Central nervous system bleeding	–	2.4	<5	–	–	4	4	0
	Umbilical cord bleeding	0.5	1.8	–	–	5.2	–	–4	–
	Menorrhagia	0.3	–	–	–	–	–	–	–
	Lliopsoas muscle	–	–	–	1.78	–	–	–	–

[a]First clinical presentations
[b]51 cases had hemophilia A, while five cases had hemophilia B

The major bleeding problem throughout the patient's life is hemarthrosis that requires immediate treatment. Bleeding into the joint leads to synovitis, causing increased proliferation and vascularization. Repeated bleeding episodes, causing a hot swollen joint, which resulted from the synovial hypertrophy and, with further bleeding, muscle weakness and loss of joint stability. This cycle of bleeding leads to erosion of joint cartilage, resulting in arthritis and the paralyzed, disfigured limb of patients with hemophilia [2, 43, 34].

4.4.1 Molecular Basis

FVIII gene that encodes FVIII protein is one of the largest genes located on the long arm of chromosome X (Xq28 position). *FVIII* gene contains high GC content and within the 9.1 kb coding region, includes 25 introns and 26 exons organized in several domains, A1, A2,A3, B and C1–C2 [45]. Exons are ranged from 69 bp (exon 5) to 3.1 Kb (exon 14) in size. The FVIII precursor protein of 2351 amino acids is encoded by FVIII mRNA which is 9.1 Kb in size [46].

4.4.2 Factor VIII Gene Mutations

The sequence of the *FVIII* gene was published in 1984, and since that time, a large number of mutations that cause hemophilia A have been identified [35].

FVIII deficiency is caused by a wide spectrum of mutations, which occur along the entire length of the *FVIII* gene. The mutations can cause quantitative or qualitative defects. In quantitative, there are defects in the level of transcription or translation and in qualitative defects, changes of individual amino acids in FVIII protein occur. Severe hemophilia is typically caused by inversions, deletions, insertions, missense mutations, and nonsense mutations. Milder forms of hemophilia are usually caused by missense mutations, while single nucleotide deletions or splicing errors also may occur [21]. There are about 70 CpG dinucleotides within *FVIII* gene. This results in to hyper-mutation status, and approximately 30% of variants are usually novel [34]. Human gene mutation database (HGMD) has reported that there are 2320 mutations within the *FVIII* gene [36]. The most frequent gene defect is the intron 22 inversion, which leads to hemophilia A with a frequency rate of 52% among individuals with severe hemophilia A [29]. The second most frequent mutation is the intron 1 inversion with a prevalence of about 1–5% among hemophilia A patients (Table 4.4) [37, 38].

The remaining cases are caused by numerous mutations spread all over the gene. As outlined in the FVIII HAMSTeRS (homology, mutation, structure, testing, and site resources) mutation database, there are over 1209 mutations in *FVIII* coding and untranslated regions. Most of these mutations are point mutations or small rearrangement. Most of the missense mutations, which are detected in mild to

Table 4.4 Frequency of factor VIII gene mutation [34, 38]

Type of mutation	Frequency percentage	Most reported relevant type of disease
Intron 1 and 22 inversions	29%	Severe
Large deletions	7%	Severe
Small deletions	6%	Severe
Non sense mutations	8%	Severe
Insertions	2%	Severe
Duplications	3%	Severe
Missense mutations	45%	Severe

moderately severe hemophilia A, are located within the exons coding for the three A domains or the two C domains [39, 40].

4.4.3 Intron 22 Inversion

About 40–50% of patient with severe hemophilia A show intron 22 inversion of FVIII, the most frequent inversion mutation in severe hemophilia A [41]. The intron 22 of the *FVIII* gene contains a 9.5 kb region, which is present outside of the gene, near the telomere of the X chromosome in two additional copies; these are termed *int22h2* and *int22h3*. *int22h1* is the sequence in intron 22 that includes the *FVIII* gene [42, 43]. The sequence identity of the three regions is 99.9%. An inversion of the *FVIII* gene can be caused by an intrachromosomal homologous recombination between *int22h1* and one of its two telomeric copies. This inversion occurs almost particularly in the male germ cells because the proposed intrachromosomal recombination would be probably inhibited by pairing Xq with its homologous in female meiosis (Fig. 4.5) [44].

4.4.4 Insertions and Deletions in the Factor VIII Gene

Insertions and deletions that cause severe hemophilia can be large (more than 50 bp) or small, usually one or more nucleotides. Insertions and deletions of a single nucleotide lead to a shift of the reading frame and thus, cause severe form of the disease. Several types of repetitive sequences, including SINES and LINES, which are present in the *FVIII* gene, may be mutated. Hemophilia in such patients is somewhere on the border between the severe and moderate forms. In severe hemophilia A, gross gene alterations (including large deletions or insertions, frame shift and splice junction changes, and nonsense and missense mutations) of FVIII account for approximately 50% of mutations detected. The most recent update of the HAMSTeRS database listed more than 200 small deletions (50 nucleotides or less than) and more than 80 insertions in hemophilia A [45–47].

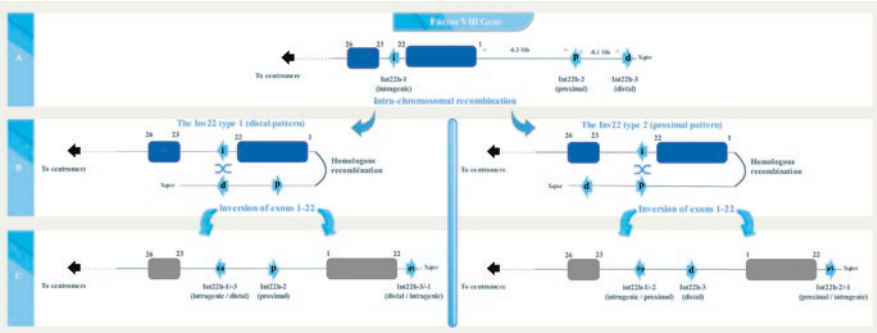

Fig. 4.5 The intron 22 inversion of the FVIII gene. Intrachromosomal homologous recombination between inverted repeats on the X chromosome account for about half of severe hemophilia A cases. Identification of a third rearrangement at Xq28 that causes severe hemophilia A as a result of homologous recombination between inverted repeats. (**a**) Show normal alleles, (**b**) Show the intra chromosomal homologous recombination, (**c**) Show the Inv22 type 1 (distal pattern) and type 2 (proximal pattern). Functional F8 is indicated by blue arrows, while non-functional F8 sequences are indicated by Gray arrows. (*Xqter* The end of the long arm of the X chromosome, *Int* intron, *FVIII* Coagulation Factor VIII, *i* Int22h-1 (intragenic), *P* Int22h-2 (proximal), *d* Int22h-3 (distal))

Genetic defects can affect the interaction sites of FVIII with other clotting factors such as FIX or VWF and cause hemophilia A [9, 10].

4.4.5 Diagnosis

Diagnosis and management of hemophilia A requires precise laboratory assessment of patients, especially precise determination of FVIII:C level. FVIII:C assay can be used for precise classification of disease, determination of the dose of replacement therapy and pre-operative management, and therapeutic response in patients with hemophilia A. Since FVIII is a cofactor and not an enzyme, FVIII assays use an indirect method performed by clot or chromogenic-based methodologies. Different methods are available for FVIII one-stage and chromogenic assays. Each of these methods has its own advantages and disadvantages. Large variation in FVIII concentrations measured in different laboratories is observed. This variation can be more profound with the use of new recombinant modified FVIII products.

4.4.6 Chromogenic Assay

The chromogenic assay uses a principal similar to the two-stage clot-based assay. In the first stage, activated FX (FXa) is generated and subsequently during the second stage, the amount of FXa is determined. In the first stage, patient's plasma is incubated with FIXa, excess FX, thrombin, calcium, and phospholipids. In this step,

activated FVIII (FVIIIa) with FIXa generates the tenase complex (FIXa–FVIIIa) and converts FX to FXa. In the second step, FXa cleaves the chromogenic substrate and the intensity of generated color is directly proportional to the amount of FXa and therefore the amount of FVIII in patient's plasma (Fig. 4.6).

The reagent consists of purified activated FIXa, purified FX, phospholipids (PL), calcium and chromogenic substrate. Added to the reagent is highly diluted patient's plasma or reference plasma as a source of FVIIIa. FX is converted to FXa and this activated coagulation factor hydrolyzes a FX specific chromogenic substrate, leading to release of chromophoric group para-Nitro Aniline (pNA). The extinction can be read on 405 nm (Fig. 4.7).

This method was recommended by the FVIII and FIX Subcommittee of International Society of Thrombosis and Hemostasis (ISTH) as a reference method

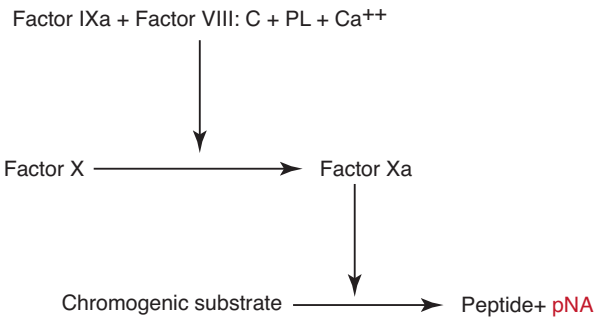

Fig. 4.6 Principle of chromogenic assay

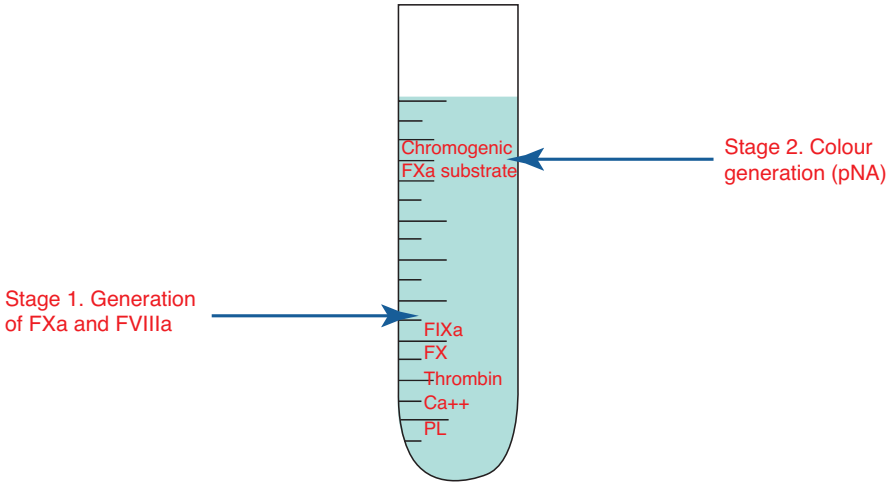

Fig. 4.7 Color generation of FXa and FVIIIa*FVIII* Factor VIII, *FIXa* Factor IX activated, *FX* factor X, *FXa* activated factor X, *Ca* calcium, *PL* phospholipid, *pNA* para-Nitro Aniline

for FVIII:C assay in plasma and/or concentrate. Chromogenic assays demonstrate less variation between laboratories than one-stage assay (Table 4.5).

The advantages of the chromogenic assay over clot-based one-stage assays include improved precision and sensitivity to low amounts of factor, but the complexity of procedure and operator burden may increase the risk of inaccuracy and errors.

Advantages:

1. FVIIIa cannot affect the chromogenic assay as it may the two-stage assay**.**
2. Direct thrombin inhibitors (DTI) or heparin can impact the chromogenic assay less than the one-stage assay.
3. In comparison with the one-stage assay, variability of the chromogenic assay is significantly lower.
4. The chromogenic assay is not dependent on FVIII deficient plasma.

Disadvantages:

1. Falsely reduced FVIII activity may be measured when a direct FXa inhibitor is present.
2. Generally, a chromogenic FVIII assay is more expensive than a clot-based FVIII activity assay such as the one-stage assay [6, 48, 49].

Table 4.5 Characteristics of chromogenic factor VIII assay kits

Kit	Vendor	Intended use	FXa substrate	Thrombin inhibitor	Detection limit	Total CV	Advantage	Measuring range
Coatest® SP4[a]	Chromogenix, Milan, Italy	Plasma sample and concentrate	S-2765: N-a-Z-D-Arg-Gly-Arg-pNA	I-2581	<1%	Range 1–20%: 5.6 (n = 80) Range 20-150%: 5.3 (n = 80)	Rapid Applications for a wide range of automated instruments	1. 1–20% 2. 20–150%
Coatest® SP[a]	Chromogenix, Milan, Italy	Plasma and concentrate	S-2765: N-a-Z-D-Arg-Gly-Arg-pNA	I-2581	<1%	Range 1-20%: 5.6 (n = 80) Range 20–150%: 5.3 (n = 80)	1.No drug interference reported 2. Heparin concentrations 0.2 IU/mL do not interfere 3. 0.5 IU/mL gives 5% inhibition 4. Due to the high dilutions used, there is no Underestimation of FVIII activity in samples containing lupus anticoagulant	1. 1–20% 2. 20–150%
Coamatic®	Chromogenix, Milan, Italy	Plasma and concentrate	S-2765: N-a-Z-D-Arg-Gly-Arg-pNA	I-2581	<1%	Range 1–20%: 5.6 (n = 80) Range 20–150%:	Applications for a wide range of automated instruments	20–150% (0.2–1.5 IU/ml) • 1–20% (0.01–0.2 IU/ml).

Technochrom® FVIII:C	Technoclone, Vienna, Austria	Plasma	FXa-1 (pNA)	α-NAPAP	<1%	–	Stability on board 24 h Reagents may be capped and refroze 2 weeks at −20 ° C after opening. Excellent accuracy and precision Economical use For plasma samples and Concentrates Adaptation sheets for common Autoanalyzers are available	0.6–1.5 IU F VIII (60–150% of normal)
DG-Chrom FVIII	Grifols, Barcelona, Spain	Plasma	FXa-1 (pNA)	α-NAPAP		4.06%	Rapid	1–130%
FVIII chromogenic assay	(Siemens, Marburg, Germany)	Plasma	CH3OCO-D-CHG-Gly-Arg-pNA. AcOH;	α-NAPAP		–	Extended onboard reagent stability	–
Biophen	Hyphen biomed, Neuville-Sur-Oise, France	Plasma and concentrate	SXa-11 (pNA)	Thombin inhibitor	-(high range) ~10% -(low range) ~2%	FVIII deficient plasma is not required Insensitive to lupus anticoagulant	More expensive than one stage More sophisticated than one stage Difficult for automation	(Low: 0 to 25% and High: 0 to 200%).

[a]The formulation of the two kits is the same, they are optimized for different test volumes

4.5 One-Stage Assay

The most common method to measure FVIII activity is the one-stage assay, which is based on partial thromboplastin time (PTT) or activated partial thromboplastin time (APTT).

In this assay, a plasma standard and FVIII-deficient plasma are required. The plasma standard, used for assay calibration, can be made in laboratory or a commercial standard can be used. In any case, this standard should be calibrated against an international standard for FVIII. It is not acceptable to assume that pooled plasma has 100 U/dL FVIII:C. FVIII-deficient plasma can be commercially available or can be obtained from patients with FVIII level <1 U/dL as long as there is no inhibitor and liver function tests are normal.

In this assay, FVIII-deficient plasma is added to patient's plasma diluted in buffer and is mixed with equal amount of deficient plasma along with the APTT reagent and incubated for appropriate time (most often 3–5 min) at 37 °C. Then calcium is added to this mixture and the clotting time is recorded. To determine the FVIII activity in patient's plasma, the clotting time of patient's sample is compared with standard curve. The standard curve is constructed by plotting the clotting time of serial dilution of standard plasma versus FVIII activity on logarithmic/linear scale graph paper. An important issue with this assay is parallelism between standard dilutions and patient's plasma dilutions. Therefore, FVIII activity assay should be performed at least on three different dilutions to check parallelism. Non-parallelism of FVIII activity will lead to inaccurate FVIII activity determination. It is generally accepted that the main reasons for non-parallelism are presence of a specific FVIII inhibitor, lupus anticoagulants, or other non-specific inhibitors.

4.5.1 Benefits of the One-Stage Methodology

1. Simplicity and readily automatable
2. The results are derived from an activity curve designed and built using clotting times of dilutions of normal reference plasma and specific factor VIII-deficient plasma.

4.5.2 Limitations of the One-Stage Assay

1. Lipemia will likely affect the one-stage assay than the chromogenic assay.
2. This method can also be influenced by anticoagulant drugs, specifically heparin, direct thrombin inhibitors, or direct FXa inhibitors.
3. If FVIII is converted to the FVIIIa during sample collection, the one-stage assay will demonstrate a false increase in FVIII activity.
4. The one-stage assay may be non-optimal for detecting an increased level of FVIII as shown by CV.
5. Finally, due to the presence of different APTT reagents, various analyzers, calibration standards, and factor deficient plasmas, there is a significant difference between one stage FVIII tests performed in different laboratories [47].

4.5.3 Discrepancy Between Chromogenic and One-Stage Assays

One-stage and chromogenic assays generally give comparable results, but discrepancies can occur in a number of circumstances including some hemophilia A phenotypes related to the presence of specific mutations and with some recombinant FVIII therapeutic agents. In 30% of patients with mild hemophilia A, discrepancy between the one-stage and chromogenic assays can be observed. In approximately 5–10% of patients with mild hemophilia A, the FVIII clot-based activity assay often falls in the normal range, while chromogenic assay yields a mildly abnormally low result. In these patients, if only the clot-based assay is used, the diagnosis may be missed; thus, simultaneous use of one-stage and chromogenic assays is recommended for diagnosis of mild hemophilia A.

When missense mutations occur in the A1–A2 and A3 domain interfaces, the result of FVIII activity by the chromogenic assay is lower than the one-stage assay. This difference can be as much as two-fold or more. The basis of this discrepancy is that such mutations can decrease FVIII heterodimer and FVIIIa heterotrimer stability. These changes have minimum effect on the one-stage assay, while in the chromogenic assay, this effect increases A2 dissociation impairing FVIII stability and leading to decreased activity.

FVIII mutations around the thrombin cleavage site, FXa binding site, and VWF binding site cause lower FVIII activity in the one-stage assay in comparison with the chromogenic assay. These mutations cause impaired FVIII activation by thrombin and impair its binding to VWF and FXa. In the one-stage assay, physiological concentration of thrombin is affected by mutations around the thrombin cleavage site, while in the chromogenic assay, high concentrations of thrombin and the longer time of incubation allow appropriate activation of FVIII and therefore this assay is less sensitive to these mutations.

4.5.4 Determination of Factor VIII Concentrates Potency

FVIII activity assay is important for monitoring patient's response to replacement therapy and for potency labeling of different FVIII products. Discrepancies were observed between FVIII activity assay by one-stage assay and chromogenic assays in the laboratory assessment of some long-acting recombinant FVIII products. One-stage assays are more sensitive to PEGylation than chromogenic assays, for example, N8-GP (Novo Nordisk A/S, Bagsværd, Denmark) a PEGylated, recombinant FVIII (rFVIII), causes the clot-based assay to be falsely elevated. The PEG moiety of this product, interferes with some particular APTT reagents, especially silica-based. Ellagic acid and polyphenol APTT reagents are not sensitive to PEGylated products and can be used for potency measurement of PEGylated rFVIII. The chromogenic FVIII assay is certified for potency testing of this product. Although one-stage assay can accurately determine FVIII activity in most plasma-derived FVIII products, they result in 20–50% lower FVIII activity than the chromogenic assay in B-domain deletion (BDD) rFVIII products. For full-length rFVIII products, the chromogenic assay yields 8–20% higher FVIII activity than the one-stage assay, which can be clinically significant [50–57] (Table 4.6).

Table 4.6 Compare the potency of FVIII replacement products

Product	Nature of product	Company	The certified potency of	Half life	Reference
N8-GP	A PEGylated, B-domain truncated derivative of recombinant FVIII (rFVIII), turoctocog alfa pegol	Novo Nordisk A/S, Bagsværd, Denmark	Chromogenic assay	19 h	[50]
NovoEight (turoctocog alfa)	rFVIII B-domain Truncation	Novoeight, Novo Nordisk A/S, Bagsværd, Denmark	One-stage clotting assay or the chromogenic substrate assay	10.8 h (in children is about 29% Shorter than in adults)	[50]
Advate®	Full-length rFVIII	rFVIII, Advate_, Baxter Bioscience, Deerfield, IL, USA	One-stage clotting assay	8.7–25.2 h	[51]
BAY 94-9027	K1804C directed PEGylation	Bayer, Leverkusen, Germany	One-stage FVIII assays, with ellagic acid APTT reagents	19 h	[52]
ReFacto	B-domain deletion (BDD) rFVIII	Pfizer, Sandwich, UK	One-stage clotting assay	14.8 ± 5.6 h (range 7.6–28.5 h)	[53, 54]
Efmoroctocog alfa	rFVIII-Fc fusion Protein	Eloctate®; Biogen Idec Inc., Cambridge, MA, USA	One-stage clotting or chromogenic assays	1.48 to 1.56-fold greater than that of RFVIII	[55]
BAX 855	PEGylated full-length rFVII	Baxter, Deerfield, IL, USA	Chromogenic FVIII assay.	1.4- to 1.5-fold increased half-life compared to Advate	[56]
CSL627	Single-chain variant of B-domain deleted FVIII	CSL Behring, King of Prussia, PA, USA	Chromogenic assay	13 h	[57]

For labeling of a FVIII product, both methods are acceptable when the results agree, but when a significant discrepancy is observed between one-stage and chromogenic assays, the more accurate method should be used. Although the one-stage assay is the most commonly used method, the advantages of the

chromogenic assay in accurately measuring some of the new modified products will increase its role in potency assignment and probably also in clinical monitoring in the future.

4.5.5 Treatment

In the 1960s and 1970s, the advance of factor concentrates, self-infusion method, and hemophilia treatment centers improved the quality of life in patients with hemophilia.

However, by the 1990's, almost 90% of severe hemophiliacs were infected with human immunodeficiency virus, acquired immunodeficiency syndrome (HIV/AIDS) [58]. Today, as a result of advances in the eradication of infectious agents in clotting factor concentrates, almost all hemophilia patients 30 years of age or younger are HIV-negative and Hepatitis C negative. Although hepatitis C infection continues as a complication of therapy, several strategies should be taken to help prevent infection. First preventive therapy, such as therapeutic abortion or vaccination against hepatitis in the prenatal and post-natal periods respectively; second, aggressive new treatment such as prophylaxis; and third, improved surgical techniques such as arthroscopic synovectomy, and finally better rehabilitation techniques [2].

4.5.6 Replacement Therapy

The traditional and common way to treat severe hemophilia A is regular supplementation with intravenous recombinant or plasma derived FVIII concentrate FVIII. However, some people with severe hemophilia and more often those with moderate and mild hemophilia are treated only as needed (also known as on demand). In children, to minimize frequent traumatic intravenous cannulations, an easily accessible intravenous port (Port-A-Cath) is often used [59]. These devices eliminate problems of finding a vein for infusion, which may be required, several times in a week. Therefore, such ports make prophylaxis programs easier. Studies have demonstrated a risk of clot formation at the tip of the indwelling catheters with high risk to become infected [60]. Individuals with mild hemophilia often are managed with desmopressin, a vasopressin analog that causes the release of stored FVIII from blood vessel walls [61].

4.5.7 Treatment of Hemarthrosis

Symptom relief, prevention of the progression of joint damage, and maintenance of function are the physician goals in hemophilic arthritis. These are similar to the goals in inflammatory arthropathy. In the subject with degenerative changes, function would be corrected by surgical/physical methods with adequate hemostatic

coverage [4]. These are similar to inflammatory arthropathy. A recent study concluded that if hemophilic patients have nonbleeding arthritis and extra-articular findings, physicians should be alert on the coexisting inflammatory disorders such as familial Mediterranean fever [62].

4.5.8 Management of Bleeding

The first choice for prevention of joint disease is prophylaxis with factor concentrates, as recommended by both the World Health Organization (WHO) and the World Federation of Hemophilia (WFH). The achieved level of the factor activity must be greater than 1% for the maximum profit. This can usually be achieved by giving 25–50 IU/kg of FVIII three times per week [63].

Extended half-life factor concentrates and subcutaneous non-factor replacement therapies have recently been used successfully to prevent bleeding in hemophilic patients [64].

4.5.9 Adjunctive Management

Analgesics such as paracetamol/acetaminophen can be prescribed to treat pain by joint bleeding, but milder opioid painkillers are more frequently used in Hemophilic joint pain.

4.5.10 Anti-Inflammatory Treatment

To treat chronic synovitis, some experts recommend intra-articular corticosteroid administrations.

4.5.11 Rest, Ice, Compression, and Elevation (RICE)

This strategy may be useful to relieve minor pain, but prolonged rest may cause motion limitation and muscle atrophy [65].

4.5.12 Physiotherapy

Physiotherapy is an important way to preserve or return movement and function to the joints. It also reduces swelling and pain, prevents injury, and maintains muscle strength [66].

4.5.13 Joint Aspiration

In hip hemarthrosis and other major and painful hemarthroses, aspiration can be prescribed and should be performed immediately after a bleeding episode (<12 h) [66].

4.5.14 Surgical Treatments

When conservative therapies fail, open surgical procedures are often the best or even the only way to cure patients with severe joint disease. The advantages of surgery must be more than the potential risks, particularly in patients with severe hemophilia and/or inhibitors.

4.5.15 Joint Debridement

Joint debridement is a surgical procedure that removes synovitis in young patients to prevent or delay articular arthroplasty [67].

4.5.16 Joint Arthroplasty

To reach the normal quality of life in patients with severe joint pain joint, replacement surgery, which also is known as arthroplasty, could be considered. Results from the most commonly replaced joints are generally good or excellent [68].

4.5.17 Fusion (Arthrodesis)

Fusion is also known as arthrodesis. It comprises joint removal and fusion of the bones. Ankle arthrodesis is carried out today and allows other joints in the foot to move normally so the patient can have a close-to-normal gait [68].

4.5.18 Treatment in Hemophilia Carriers

Most carriers are asymptomatic, but, if they are subject to trauma or surgical intervention, they may show prolonged bleeding. In these instances, treatment must be initiated using the same strategies as in patients with hemophilia. Pre-existing clotting factor level and the cause and severity of the bleed determine the dosage and duration of treatment [27].

4.5.19 Problems Related to Treatment of Hemophilia

Viral infections and immunization against the FVIII protein are the most reported problems related to treatment of patient with hemophilia. The main issue with factor replacement therapy is the development of inhibitor antibodies against FVIII. As the patient does not have its own copy, it recognizes the "normal form" of FVIII as foreign. Due to antibody development, FVIII infusions become ineffective. Recently, FVIIa has become available as a treatment for hemorrhage in patient with hemophilia with FVIII inhibitors [64, 69]. An inhibitor may be suspected when unexpected bleeding in the response to treatment is not stopped by factor substitution as quickly as it should. After suspicion, the inhibitor may be discovered during routine screening. As a follow-up, its titer must be determined. Inhibitor titer levels are important as a suitable treatment is based on the patient's inhibitor titer.

4.5.20 Viral Infections

Adults with hemophilia have one of the highest prevalence of hepatitis C virus (HCV) infections among all populations at risk of HCV infection. Prior to 1987, approximately all patients treated for hemophilia with plasma products were exposed to HCV, and more than 80% had chronic infections. In one study, of the 350 patients with hemophilia A and B, 232 individuals (about 66%) had been infected with HCV. There was no independent risk factor for infection based on multivariate logistic regression model [70]. The prevalence of HCV infection among HIV-infected patient with hemophilia was found to be 66%. Of the 31 patients with HCV/HIV, 21 persons (67.7%) had no history of transfusion. The authors noted that contaminated FVIII (clotting factor) could be responsible for disease acquisition [71].

4.5.21 Inhibitor

A FVIII inhibitor is a polyclonal high-affinity immunoglobulin G (IgG) that is directed against the FVIII protein [72]. IgG4 antibodies are the predominant antibodies that do not fix complement [72, 73]. The domains A2, A3, and C2 are the main domains that inhibitory antibodies are directed against [74].

The most dangerous complication of the hemophilia A management is the development of an inhibitor against FVIII. Based on data reported worldwide, the frequency of inhibitor development is 5–10% (totally) and 10–15% in severe patients [75].

Inhibitor development is defined as the number of new inhibitors diagnosed in a specific time period. Published articles report differences in the rate of inhibitor development likely due to disappearance of many transient low-titer inhibitors and successful tolerance of others [76, 77]. Based on different studies from around the

world, the prevalence of inhibitors in hemophilia A is greater compared with hemophilia B [78]. For example, in Saudi Arabia, patients with FVIII inhibitors were observed in 43 (29.3%) out of the 147 patients, and only 1 out of the 54 patients developed FIX inhibitor [79].

4.5.22 Inhibitor Associated Risk Factors

The following are the main risk factors for inhibitor development [74, 80].

1. Number of exposures to clotting factor concentrates
2. Increased dosage of clotting factor concentrations
3. Spanish ethnicity or black race
4. The positive history of inhibitors in family
5. High risk mutations such as multiple exon deletion or stop codon

4.6 Inhibitor Evaluation

The Bethesda assay is used to determine the presence of a factor inhibitor and to measure its titer. This assay uses dilutions of patient plasma with normal pooled plasma (NPP) and also control dilutions containing buffer and NPP. Following incubation, the remaining FVIII activity in the patient sample is compared to that of the control. The percent of residual FVIII activity in the patient mix is then converted to Bethesda units (BU) using an equation or read from a graph. One BU is defined as the amount of inhibitor producing a residual activity of 50% [81]. In the Nijmegen modified assay, the NPP that used in patient and control mixtures are buffered with Imidazole, and control tube contains FVIII-deficient plasma instead of buffer [81, 82]. This improves FVIII lability and helps prevent falsely elevated Bethesda results.

4.6.1 Treatment in Patient with Inhibitor

The healthcare costs associated with inhibitor development can be worrying because of the amount and type of treatment products required to stop bleeding in such patients. In addition, these patients are much likely to be hospitalized for a bleeding complication, and they are at increased risk of lethal bleeding complications [83–85].

4.6.2 The Treatment Lines

Following guidelines such as “treatment of bleeding in the presence of inhibitors” is usually recommended for patients with hemophilia with inhibitor [78].

4.6.3 Treatment of Bleeding in the Presence of Inhibitor

In persons with low-responding inhibitors and low Bethesda titers (less than 5 units), therapy with further FVIII replacement products is usually possible. In these cases, higher doses of the factor and/or more frequent (or continuous) factor infusions may be useful to overcome the effect of the antibody. However, when there is a large titer of antibody (at least 5 BU), higher doses of therapeutic FVIII or IX is usually not effective because the inhibitor can neutralize even large doses of factor. Plasmapheresis or immune absorption may be useful in eliminating inhibitors, especially when life-threatening bleeding is treated with an excess factor but a high level of inhibitor exists. However, as soon as the patient receives the factor, high concentrations of antibody develop within several days. Another approach is bypass therapy, which includes various therapeutic products such as prothrombin complex concentrate (PCC), activated PCC (aPCC), and recombinant activated FVII (rFVIIa). Therapeutic choice is based on circumstances such as inhibitor characteristics, nature, and severity of the bleed, age, and treatment response pattern of the individual (Table 4.7) [78].

Immune Tolerance Induction (ITI) Therapy: The purpose of ITI therapy is to halt the inhibitor production by the immune system of individuals with hemophilia. This method teaches the body to accept factor as a normal part of blood. In this way, patients receive more frequent, low dose factor [86]. People with low titer inhibitor (<5 BU) may be treated with higher amount or more frequent factor concentrate. Based on the inhibitor status of the patients, immune tolerance induction has been successfully used for years. High-dose (200 IU/kg/day) and low-dose (50 IU/kg × 3 times/week) ITI regimens are both effective at eliminating the inhibitor. However, bleeding manifestations are common during the administration of low-dose ITI. This bleeding problem has recently been overcome by using emicizumab prophylaxis in conjunction with low-dose ITI or reduced frequency of factor administration in a prospective, multicenter, phase IV study [87].

4.6.4 Bypassing Therapy Products

Bypassing agents, which can stimulate the coagulation cascade without the requirement for the desired factor, are used to treat bleeding complications in people with high-level inhibitors (>5 BU). Autoplex® was the first activated prothrombin complex concentrate (APCC) product. Despite the unknown mechanism of action for bypassing FVIII, Autoplex® appears to be a useful and necessary interim product that is safe and effective. To avoid thrombosis, use of fibrinolytic inhibitors at the same time must be avoided [88].

Today, the most well-known bypassing agents, such as activated prothrombin complex concentrate (APCC) (FEIBA®, anti-inhibitor coagulant complex, Shire, Dublin, Ireland) and recombinant factor VIIa (rFVIIa (NovoSeven®, eptacog alfa, NovoNordisk, Bagsvaerd, Denmark), are used to treat and prevent bleeding in hemophilic patients with inhibitors [89]. Factor eight-inhibitor bypass activity

Table 4.7 Treatment of bleeding in patients with hemophlia A and inhibitor

Inhibitor titer	Approach	Advantages	Disadvantages
<5 Bethesda units	Higher doses of the factor and/or more frequent, or continuous	May be useful to overcome the antibody	It may trigger stronger immune response and increases inhibit titer
≥5 Bethesda units with life-threatening Bleeding	Plasmapheresis or immune adsorption then factor replacement	Eliminate the risk of bleeding or lethal episodes	After the patient receives, the factor makes large amounts of new antibody within several days.
≥5 Bethesda units without is life-threatening Bleeding	Bypass therapy with (PCCs)	Bypasses the requirement for factor	As the same as APCC
	(APCC) with doses of 50–100 units/kg are every 8–24 h, depending on the severity of the bleed	Effective for 60–90% of musculoskeletal bleeds, major and minor surgery prophylaxis	1. Short-acting 2. Paradoxically causes either more bleeding or excess clotting. 3. Worsens problem if antifibrinolytic drugs are used along with it 4. Contains small amounts of FVIII and stimulates new antibody production
	Factor VII (rFVIIa). Multiple doses of 90 units/kg or more infused every 2–6 h may be required	1. Bypasses the requirement for factor 2. Effective in the prevention and treatment of joint hemorrhage, life-threatening bleeding and surgical bleeding 3. Contains no FVIII. So doesn't restimulate antibody Production	1. Short-acting 2. Excess blood clotting
	Immune tolerance induction (ITI) infusions of variable doses of FVIII or IX administered for a period of weeks to years in an effort to tolerize the immune system	Accept treatment with the missing clotting factor without producing further antibodies	Time-consuming and costly

Consequently, factor VII (rFVIIa) is frequently the bypass therapy of choice for patient with hemophilia

(FEIBA®) is also an anti-inhibitor coagulant complex, which is indicated in patients with hemophilia A and B inhibitors [90].

- Control and prevention of bleeding episodes.
- Perioperative management.
- As the prophylaxis to prevents or decreases the rate of acute bleeding.

A recent study investigated the efficacy and side effects of a new class of therapeutic drugs for hemophilia patients who have inhibitors that act by enhancing coagulation (emicizumab®) and inhibiting anticoagulant pathways (fitusiran® and concizumab®) [91]. Emicizumab is a licensed bispecific antibody that mimics factor VIII. A novel bispecific antibody that binds factor IX and factor X, partially mimicking the functional property of factor VIII in the tenase complex, has been shown to be highly effective in treating antibodies to factor VIII.

4.6.5 Salvage Treatment

Patients with hemophilia who have high inhibitor titers and persistent bleeding after major surgery may benefit from sequential bypassing agent therapy. In a previously reported case, this complication was controlled by sequential administration of recombinant factor VIIa and activated prothrombin complex concentrate [92].

4.6.6 Mouth Care in Hemophilia

For patients with severe hemophilia, factor replacement therapy is necessary before surgery or regional block injections. Some studies proposed a dose of 50 international units per kilogram of body weight (IU/kg) of FVIII before a tooth extraction in patients with hemophilia. The safe and cost-effective method to control bleeding of teeth extraction is the use of fibrin glue and anti-fibrinolytic agents such as tranexamic acid.

Lifestyle can be significantly responsible for gum diseases. Smoking and tobacco use may contribute to periodontal disease and oral cancer. Oral hygiene is the best way to prevent caries and gum diseases such as chronic periodontitis gingivitis [33, 93].

4.6.7 Bleeding Prevention Strategy

Reducing the frequency and amount of sugars in the diet, avoiding smoking, using fluoride, mouth protection and soft plastic mouth guards may be needed to prevent damage from trauma, tooth grinding (bruxism), and acid erosion in individuals with hemophilia [33, 94].

4.7 Gene Therapy

The most promising curative strategy in hemophilia is gene therapy. To be successful, the missing or defective clotting gene must be identified and replaced by a normal gene fragment, which is inserted in the genetic material of cells of the

hematopoietic system, liver, skin, muscle, or blood vessels. Because the *FVIII* gene is very large and difficult to insert compared with the *FIX* gene, hemophilia B was the first candidate to be treated by gene therapy. Several studies using test tubes and animal studies predicted that gene therapy would be effective. Recently, the FIX gene was successfully administered to the dogs with FIX deficiency [95]. A clinical trial that began in 2010 has reported long lasting conversion of severe hemophilia B to mild status by elevating factor IX levels above 5% [96]. Subsequently five other trials in hemophilia B have started using similar vectors based on Adeno Associated Virus.

The ultimate goal of treatment of gene-deficient diseases is the elimination of disease for the lifetime of the patient. Gene therapy is the best and only way to achieve this goal, which replaces the faulty gene sequence with the correct one. Continuous production of FVIII due to gene therapy is superior to non-continuous FVIII administration. This challenging task has now been accomplished. The first clinical trials attempting to treat hemophilia with gene therapy primarily demonstrated good safety, but without good efficacy. The next efforts, which re-engineered the vector plasmids and delivery systems, resulted in markedly improved outcomes in animal models of disease. Retroviral vectors that can permanently insert the *FVIII* gene into the DNA of the host cell appear the most suitable for this specific purpose (Fig. 4.8) [97, 98].

We divide the gene therapy strategies into two groups:

1. Gene replacement by an ex vivo or in vivo approach
2. Other proposed approaches

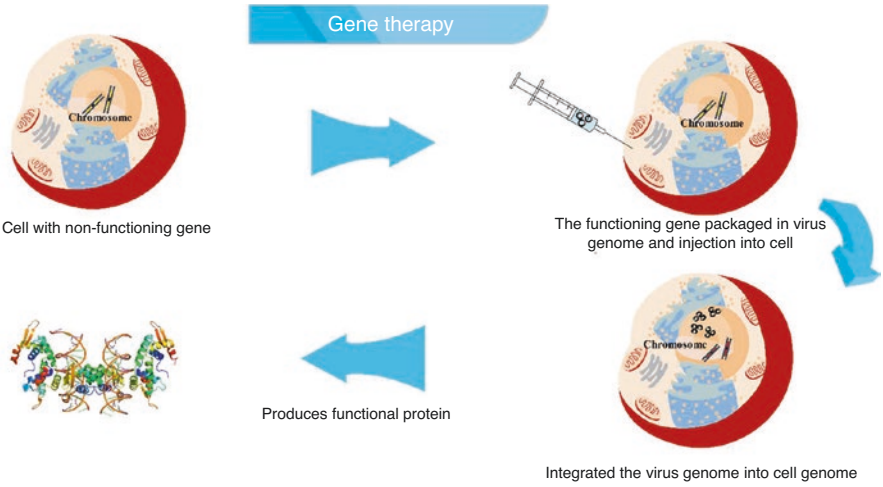

Fig. 4.8 The in vivo approach of gene therapy. The target gene packaged in virus genome, then delivered in a body organ such liver. The virus genome is integrated into cell genome and produces functional protein. (*ITR* Long terminal repeats, *PA* Palindromic repeat)

4.7.1 The Gene Replacement by an Ex Vivo or in Vivo Approach

Ex vivo and in vivo treatments for hemophilia gene therapy have been proposed. Ex vivo gene therapy involves isolating the cells from the patient, expanding and genetically modifying them in a culture medium with vectors which express FVIII, and then transferring the transfected cells to the patient's liver, skin etc. Different cell types such as skin fibroblasts, endothelial cells, epithelial cells, myoblasts, hematopoietic stem/progenitor cells, and bone marrow (BM) stromal cells have been considered as potential targets for hemophilia gene therapy [99–101].

Another particular creative target is megakaryocytes and platelets. An ex vivo hemophilic treatment gene that uses megakaryocytes and platelets has shown to be an effective homeostatic response in standard hemophiliacs [102, 103]. The initial cell capacity for transferring FVIII to the bloodstream is highly dependent on the site of implantation. The liver appears to be a suitable target for hemophilia gene therapy. FVIII produced in the transduced hepatocyte should have ready access to the circulation. In addition, the stability of FVIII increases when it is secreted in an environment with access to VWF either in the circulation or VWF produced in the sinusoidal endothelial. In contrast, coagulation factors produced in non-hepatic tissues might not be properly processed due to differences in post-translational modification. Ex vivo gene therapy is based on the use of viral vectors (especially retrovirus) and sometimes in non-viral transfer systems (Table 4.8) [104].

The in vivo gene therapy is more affordable than ex vivo. However, the host immune response towards the viral vector is the major drawback of in vivo gene therapy. The gene delivery system in the gene therapy should be efficient, safe, non-immunogenic and allow for long-term gene expression. Most importantly, the comparison between gene therapy for hemophilia A with existing protein replacement

Table 4.8 Viral-based-vectors used for gene therapy of factors VIII deficiency

Vector	Nucleic acid	Advantages	Disadvantages
Retroviral	RNA	Efficient transduction genomic integration persistent expression	Oncogene derivation random insertion cell division-dependent (except for lentiviral)
Adenoviral	DNA	Transduces non-dividing cells accommodates large cDNAs high level of don't have chronic toxicity	Immune responses to AV episomal (no integration) transient expression
Adeno-associated viral	DNA	Integration (partial) persistent expression different serotypes	Limited size of cDNA possible rearrangements pre-existing anti AAV antibody able to prevent successful transduction liver disease in patient especially HCV positives Immune response to the capsid

AV Adenoviral, *AAV* Adeno-associated viral, *HCV* Hepatitis C virus

therapies must be in favor of gene therapy [105]. Retroviral, lentiviral, adenoviral, and adeno-associated viral (AAV) vectors, each with their own benefits and limitations, are the most widely used vectors in hemophilia gene therapy. Moloney murine leukemia virus (MoMLV)-based retroviral vectors are the first and the most commonly used vectors for clinical trials [106]. Although non-viral vectors are less immunogenic than viral vectors, the viral vectors are the most chosen method since it is more manageable clinically (Table 4.7) [105]. If the immune system is stimulated, the treatment strategy includes immune-modulating drugs. For instance, inhibition of poly (ADP-ribose) polymerase-1 (PARP-1) and nuclear factor kappa B (NF-κB) signaling synergistically down regulates the immune response against recombinant AAV2 vectors. A combination of non-viral vector systems with some viral components can combine the benefits of both systems. The *FVIII* gene can be cloned, but this is too large to be packaged efficiently into any commonly used gene therapy vectors. However, there is a full-length recombinant Pegylated FVIII [107]. The B-domain deleted FVIII (FVIII-BDD) cDNA had been shown to express a higher level of FVIII protein as compared to the full-length FVIII cDNA and is suitable for the restricted packaging limits of vectors [105]. The packaging capacity of AAV is about 5 kb, but the FVIII expression cassette is about 7 kb. The use of a FVIII-BDD (4.37 kb in size) overcomes this challenge, but constrains the size of included regulatory elements that control FVIII expressions [108]. There are several FVIII-BDD products available: BAY94-9027, NovoEight, Eloctate, and others [107]. Studies in hemophilia A dogs that had developed inhibitors have demonstrated that inhibitors could be eradicated following AAV-mediated, liver-directed gene therapy [109]. Proprotein convertases are a family of proteins that activate other proteins by proteolytic activity. One member of this family is Furin [110]. When Furin processes FVIII-BDD, its procoagulant activity is degraded. This process also reduces its secretion. Therefore, inhibition of Furin leads to secretion of FVIII-BDD in mammalian cells [111]. Current challenges of gene therapy include ensuring long-term stable protein expression, vector immunogenicity, the potential risk of insertional mutagenesis, and hepatic toxicity (Fig. 4.8) [112].

4.7.2 Advantages of Lentivirus (LV) Transduction

1. Can result in a long-term therapeutic effect.
2. Some evidence suggests that a single treatment of LV-mediated gene therapy may be sufficient for life-long effect.
3. LV can transduce both non-dividing and dividing cells, and this is the most important advantage of LV in comparison with traditional retroviral vectors. This leads to a considerable increase in LV efficacy in the targeting of primitive stem cells.
4. Self-inactivating (SIN)-LVs provide improved safety by reducing transactivation capacity [113].

4.8 Adeno Associated Virus Gene Therapy for Hemophilia A

Recently, a trial of AAV gene therapy has reported that patients with severe hemophilia A treated by peripheral vein infusion of a single dose of vector achieved and have maintained normal level of factor VIII for more than a year. However, pre-existing AAV antibody is one of the most significant barriers to gene therapy in hemophilic patients. Pre-existing antibody seroprevalence, for example, was observed to be highest in Turkey [67% in Turkey, followed by patients from Dutch (27%) and Italian (14%) referral institutions]. This increased prevalence may reduce the number of potential candidates in hemophilic patients for upcoming gene treatments in some countries [114].

4.8.1 Other Proposed Approaches

1. Aminoglycoside antibiotics such as gentamycin can transiently suppress the nonsense mutations at the ribosomal level in a patient with hemophilia. This could potentially convert severe hemophilia to a moderately severe phenotype; however, this intervention has failed to show a clinically significant effect [115].
2. At the RNA level, trans-splicing could potentially be feasible and effective to correct hemophilia inducing inversions in mice [116].
3. Some prokaryotes like bacteria and even archaea use the Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR and CRISPR-associated (Cas) system in their immune system. This provides the CRISPR/Cas9 genome editing technology that allows for permanent changes in the genes of organisms [117]. This process leads to the creation of a double strand break (DSB) at a distinctive region of the genome. Then The DSB(s) are typically repaired by an error-prone path, which is named non-homologous end joining (NHEJ), or error-free path, which is known as homology-directed repair (HDR). Thus, the HDR pathway could be used to correct the monogenetic diseases including hemophilia after CRISPR-induced-DSB. However, it has been reported that gene correction with NHEJ is also possible [118].

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