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Hemophilia: Genetics, Diagnosis and Treatment

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Abstract

Hemophilia A and hemophilia B are hereditary X-linked disorders of blood coagulation caused by a deficiency of factor (F) VIII or FIX, respectively. Affected males suffer from joint and muscle bleeds and other serious internal bleeding, the severity of which is correlated with the level of the coagulation protein in their blood. Early diagnosis and cloffing factor (CF) replacement therapy has remarkably improved the outlook of patients with hemophilia, so that they can live near normal lives. However, major issues such as compliance due to need for frequent venous access and treatment failure due to development of alloantibody (inhibitors) to the replaced factor remain. Furthermore, due to cost and availability of CF3, state of the art care is inaccessible to a vast majority of patients in developing countries. Molecular genetic studies of the FVIII and FIX genes have not only allowed better understanding of the disease and its diagnosis but also led to the development of recombinant therapeutic products as well as gene therapy. Genetic evaluation is also becoming increasingly important for predicting the development of inhibitors, apart from carrier detection and genetic counseling.

Keywords: Hemophilia; Phenotype; Genotype; Diagnosis; Therapy

Introduction

Blood clotting is a host defence mechanism that in parallel with the inflammatory responses helps not only protect the integrity of the vascular system but also promotes repair after tissue injury. This process involves a series of orderly steps involving components of the vasculature, platelets (primary hemostasis) and coagulation proteins (secondary hemostasis) that leads to the formation of platelet plug and culminates in the formation of a stable fibrin clot. Congenital defects of platelets or plasma proteins involved in this process generally lead to bleeding disorders [1,2]. In some of these disorders, patients with severe disease are prone to spontaneous bleeds with critical consequences. This situation occurs more commonly in hemophilia A and hemophilia B, both of which are X-chromosome linked and caused by a defect of coagulation factor (F) VIII or FIX, respectively [3-5]. Males (46:XY) are affected by the disease while females (46:XX) are carriers. This article will describe the clinical and diagnostic aspects of hemophilia and discuss how the knowledge of molecular genetics of FVIII/FIX has contributed to improved patient care.

Role of factors VIII and IX in coagulation

The FVIII and FIX proteins circulate as inactive precursors in circulation and are activated only during a hemostatic challenge during the "propagation phase" of the coagulation cascade (Figure 1). While FVIII is a cofactor with no enzyme activity, FIX is a potent serine protease that requires activated FVIII as a co-factor for its function. Upon activation, and in the presence of calcium ions and phospholipids, FVIII and FIX form an active "tenase complex" (FX activating complex) on the surface of activated platelets [6]. The co-localization of three receptors (for the enzyme, FIXa; the substrate, FX; and the cofactor, FVIIIa) results in a 24 million-fold acceleration of the rate of FX activation and the subsequent generation of sufficient amounts of thrombin to effect hemostasis [7]. In a patient with hemophilia, the initiation phase of blood coagulation would proceed normally with the formation of small amount of thrombin necessary for initiating secondary hemostasis. However, the absence of either FIX or its cofactor FVIII severely diminishes the secondary burst of thrombin generation during the propagation phase. This compromises the activity of FX activating complex, leading to inefficient fibrin formation and resultant bleeding diatheses.

Inheritance

Hemophilia has an incidence of 1 in 5000 (Hemophilia A) and 1 in 25000 (Hemophilia B) males, respectively. No ethnic or geographic predisposition has been noted [8]. There is a 50% chance that a carrier mother will transmit the defective X-linked gene to the male or female child. All female offsprings born to a hemophilic father are obligatory carriers [9]. When more than one affected patient in the family exists the inheritance is termed "familial hemophilia" while "sporadic hemophilia" results from *de novo* mutations in ~30% patients. Among the latter group, ~10% of patients have mothers with somatic mosaicism who may not appear to be carriers [10]. Rare cases of hemophilia in females have been described caused by non-random X chromosome inactivation or the presence of two copies of the defective *F8* or *F9* genes [11-13].

Clinical manifestations and their pathogenesis

Hemophilia A and B are clinically indistinguishable and heterogeneous disorders [6]. Their clinical manifestations are identical, with an increased tendency for musculoskeletal, soft tissue and mucocutaneous bleeding. Bleeding into other organs also occur. The severity of bleeding symptoms correlates with the coagulant activity of the deficient factor. Thus, three clinical phenotypes (severe, moderate and mild) are recognized (Table 1) [14]. This conventional classification generally predicts the risk of (spontaneous) bleeding as well as guides management and genetic diagnosis [15].

Severe hemophilia (FVIII or FIX coagulant <1% of normal) is characterized by recurrent hemorrhages occurring spontaneously

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or after trauma and surgery. In the absence of family history, infants often present with post-circumcision bleeding. There is also a 4% risk of intracranial bleeds associated with delivery [16]. Infants with severe hemophilia typically develop palpable subcutaneous ecchymoses at 3 or 4 months of age, but significant musculoskeletal bleeding complications are usually evident by 1 year of age with the onset of walking. Large hematomas may also follow deep intramuscular injections given for vaccinations. Oral bleeding predominantly from lip and tongue biting becomes apparent by two years of age and continues into childhood with loss of deciduous teeth. By 2 to 3 years of age, bleeding into joints and muscles becomes common. Major hemorrhage can occur in severely affected individuals in any organ.

Moderate hemophilia is less often associated with spontaneous hemorrhage, but bleeding is usually precipitated by minor trauma or surgery. The first symptoms become apparent at a later age with more intense activities.

Mild haemophilia is generally not associated with any spontaneous bleeding. It presents with bleeding only after major trauma or surgery, but more often these patients are diagnosed before elective surgery when routine coagulation screening reveals abnormalities [15].

Variations in the clinical profile of severe hemophilia: Patients conventionally classified as having severe hemophilia (<1% of normal clotting activity) usually have 15 to 35 spontaneous joint and muscle bleeds per year [17-19] and account for 60-70% of all patients with

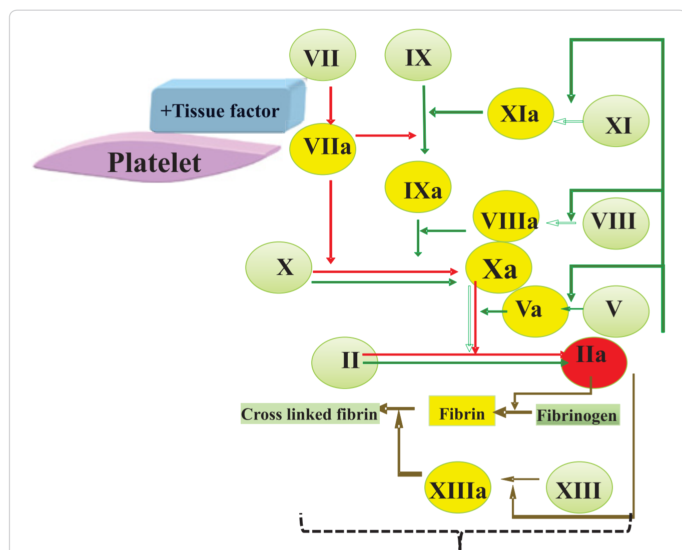

Figure 1: Blood coagulation *in vivo:* Mammalian blood coagulation is initiated by the exposure of membrane-bound tissue factor to factor (F) VII. Activation of FVII to the protease FVIIa results in the activation of FIX and FX by the TF–FVIIa complex. In the absence of its activated cofactor FVa, FXa generates only trace amounts of thrombin. Although insufficient to initiate significant fibrin polymerization, trace amounts of thrombin formed in this '*initiation*' stage (*red arrow*) of coagulation are able to back-activate FV and FVIII by limited proteolysis, leading to the explosive generation of thrombin during the '*propagation*' phase (*green arrow*) that ultimately leads to generation of a fibrin clot. During this propagation phase, FIXa and FVIIIa form the tenase complex in the presence of calcium and negatively charged phospholipid membranes to generate FXa. FXa and FVa form the prothrombinase complex to generate sufficient amounts of thrombin. This thrombin efficiently cleaves off fibrinopeptides A and B from fibrinogen, which results in the polymerization of fibrin monomers to a fibrin network. The fibrin clot is stabilized by activated FXIII (FXIIIa) (converted from its inactive form by thrombin), a transglutaminase that catalyses covalent cross-linkage of fibrinogen (*Stabilization phase, brown arrow*).

Classification	Coagulation factor level (VIII:C or IX:C)	Clinical phenotype
Severe	<0·01 IU/mL (<1% of normal activity)	Spontaneous soft tissue and musculoskeletal bleeding
Moderate	0·01–0·05 IU/mL (1–5% of normal activity)	Bleeding into joints and muscles after minor injuries or after surgical intervention
Mild	>0·05–0·40 IU/mL (5–40% of normal activity)	Post-operative and post-traumatic bleeding only

Table 1: Phenotypic classification of hemophilia and its clinical features (White et al., 2001)[14].

hemophilia. However, within this group, there is considerable heterogeneity in clinical presentation. A subset of these patients (10-15%) have clinically mild disease [15,18-20]. Variations in the bleeding frequency, age at first bleeding and extent of joint damage have all been reported by several groups [15,18-23]. Though such phenotypic heterogeneity is intriguing, only a few studies have attempted to address its basis. Contributing factors include, varying levels of FVIII:C activity (below 1%) [24,25], pharmacokinetics of the replaced clotting factor concentrate [26], the type of mutation and the concomitant presence of prothrombotic factors [27-29]. Functional polymorphisms in the *F7* (Arg353Gln), tissue factor (-1208 Insertion/deletion) and endothelial protein C receptor (23 bp insertion/deletion in exon 3) genes have also been reported to impact the phenotype of severe hemophilia [24,30,31]. It has also been suggested that polymorphisms in inflammatory cytokine genes such as TNFα may also modulate the clinical manifestations of severe hemophilia [30].

Hemarthrosis and arthropathy: Spontaneous bleeding into a joint (*hemarthrosis*) and muscle is the most frequent manifestation of severe hemophilia. Ninety percent of all bleeding episodes in patients with hemophilia occur into the joints (Figure 2) [32]. Most often this affects the knees (>50% of all events), followed by the elbows, ankles, shoulder and the wrists [32]. Knees and elbows are particularly vulnerable because they must withstand rotatory and angular stresses as relatively unsupported hinge articulations [33]. The hallmarks of hemophilic arthropathy involve joint bleeding, inflammation, synovial hypertrophy/villous formation and cartilage/bone destruction. After an acute intra-articular bleed, autolysis of erythrocytes results in the deposition of hemosiderin in the synovial tissue. This triggers inflammation characterized by elevated levels of pro-inflammatory cytokines (interleukin-6 [IL-6], IL-1β, tumor necrosis factor α) [34,35]. Within 4 days, neovascularization of the sub-synovium and focal areas of villous formation can be detected on the synovial surface, resulting in synovial hypertrophy, which is friable and more likely to re-bleed with even minimal stress, such as weight bearing or minor trauma [36].

Repeated bleeding evolves into a chronic, persistent inflammatory disorder termed "*hemophilic synovitis*". A vicious cycle of re-bleeding may become established, creating a "*target joint*". The actual mediators of cellular proliferation and synovial hypertrophy are unknown, but c-myc and mdm2 expression could be contributory along with increased evidence for the role of key angiogenic factors (vascular endothelial growth factor [VEGF], matrix metalloproteinase-9) in cellular proliferation [37]. Repeated bleeding into the target joint is associated with progressive inflammation of joint capsule and elevated concentrations of hydrolytic enzymes, such as acid phosphatase, cathepsin D and collagenases in the synovial fluid that exacerbate the proteolysis and destruction of cartilage and bone [38]. Eventually, these processes lead to complete erosion of the articular cartilage and permanent joint damage- "*the chronic hemophilic arthropathy*", characterized by compromised range of motion and pain leading onto

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contractures, articular fibrosis and progressive joint stiffness [32,39].

Muscle hemorrhage: Muscle is the second most common site (30%) of spontaneous bleeding in severe hemophilia[40]. The cause of this is unclear but may be the result of sudden stretch or unaccustomed stress. Intra-muscular hematomas typically present with localized tenderness and pain either on movement or at rest [41] and may be associated with low-grade fevers and large ecchymoses. Hemorrhages into large muscles may be quite extensive but can resolve without residual effects. In contrast, a much smaller bleed in a closed fascial compartment may cause significant compression of vital neurovascular structures with attendant distal ischemia, possible gangrene, flexion contractures and neuropathy[42]. Hematomas of the psoas muscle and retroperitoneal space are particularly problematic and produce a sudden onset of inguinal pain and compromised range of motion of the ipsilateral hip, which assumes a markedly flexed position usually without lateral rotation. Resultant damage to the femoral nerve can then affect the quadriceps muscle and thus the stability of the knee joint.

Intra-cranial hemorrhage: This is the most serious bleeding related complication of hemophilia and can be rapidly fatal. The incidence of spontaneous central nervous system (CNS) bleeds vary between 3.5-4% during the neonatal period in countries with a good standard of care for hemophilia [43]. CNS bleeds are also frequent after the neonatal period, affecting 3–10% of the hemophilia population who are treated episodically [43]. Approximately 20% of CNS events result in death [43], more than one-third of survivors develop long term neurologic sequelae [44]. CNS bleeding can be subdural, subarachnoid, intraspinal or intracerebral. The most frequent presentation of intra-cranial bleeding is headache often with vomiting, seizure and altered conciousness.

Bleeding into other tissues: Bleeding into other organs can occur spontaneously or with trauma in hemophilia. Mild mucosal bleeding is not uncommon [45]. Most of these are self-limited. Traumatic or inflammation related hemorrhage in the oropharynx may lead to life-threatening upper-airway obstruction. Epistaxis and bleeding after dental procedures are also common. Gastro- intestinal bleeding occurs in approximately 10-15% of adults with severe hemophilia [46]. This frequency rises in patients with portal hypertension due to chronic hepatitis and cirrhosis. Non-steroidal anti-inflammatory drugs may provoke gastro-intestinal bleeding. Spontaneous gross hematuria occurs in individuals with severe hemophilia. It is generally benign and painless condition unless accompanied by clots blocking the urinary tract [47]. Persistent mucosal bleeding usually signifies a local lesion that needs endoscopic or radiological evaluation. In such cases, appropriate intervention with replacement therapy or other therapeutic measures may be needed.

Molecular genetics

Factor VIII: F8 gene maps to the long arm of X-chromosome (*Xq.28*), spans ~186 kb and produces an mRNA of 9Kb [48]. FVIII is a large multidomain glycoprotein of 2351 amino acids with domain structure A1-A2-B-A3-C1-C2 [49] (Figure 3). Upon FVIII activation, the large B domain is removed by thrombin cleavage [50]. Normally, FVIII is synthesized primarily in the liver, and also in kidney, and endothelial cells, as an inactive single-chain protein. After extensive post-translational processing, FVIII is released into the circulation where it is stabilized by von Willebrand factor (VWF) and has a half-life of ~12hrs in adults. Upon activation of the coagulation cascade, FVIII is proteolytically cleaved to facilitate dissociation of VWF and development of biological activity to participate in the factor X activating complex. Activated FVIII rapidly loses its activity. This process is either through enzymatic degradation mediated by FIXa, FXa, and activated protein C[51] or by subunit dissociation as activated FVIII is intrinsically unstable [52]. Subsequently, FVIII catabolism is mediated by low-density lipoprotein receptor-related proteins (Figure 4) [53,54].

Heterogeneous mutations (~2183 in HGMD®, Human Gene Mutation Database. http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F8) including a variety of deletions, insertions, missense, nonsense and splice site mutations, apart from the common intron 1 and intron 22 inversions in the *F8* gene, have been reported to cause the clinical phenotype of hemophilia A (Table 2). The high frequency of this disorder (1:5000) is due to the high mutation rate of the *F8* gene, which ranges from 2.5 x 10^{-5} to 4.2 x 10^{-5} [55,56]. Two major effects contribute to the high mutation rate: the prevalent intron 22 inversion [57] and the size of the FVIII gene[48]. The postulated mechanism for the intron-22 and intron-1 inversion involves flipping of the tip of the X chromosome [58, 59], which is facilitated in male meiosis but inhibited by homologous pairing of the X chromosomes in female meiosis (Figure 5). In contrast, large deletions often originate from recombination's expedited by the pairing of the X chromosomes in female meiosis. For increased frequency of point mutations in *F8* gene (~50% of inversion-negative cases), the continuous replication of male germ cells and the huge size of FVIII gene represent the major determining factor. However, it must be also noted that a disease-causing mutation is not identified in the *F8* gene in ~ 5% of cases with hemophilia A [60], and efforts are underway to define the basis FVIII deficiency in these patients [60].

Factor IX: The *F9* gene is located at chromosome X (Xq27) containing eight exons and measures 34 kb in size (Figure 3) [61,62]. FIX is synthesized in the liver as a 57 Kda and 462 aminoacid pre-cofactor. The first exon encodes a signal sequence ensuring secretion from the hepatocyte and is cleaved in the rough endoplasmic reticulum by a signal peptidase; The 29 aminoacid exon 2 encodes the propeptide sequence and the *gla* domain. The propeptide provides a recognition site for interaction with a vitamin K-dependent *γ* - carboxylase which is responsible for effecting this essential post-translational modification of the first 12 glutamic acid residues in the *gla* domain of the mature polypeptide [63]. Following the *γ* carboxylation of these residues, the propeptide is cleaved from the mature protein by a specific peptidase prior to secretion to give rise to a 415 aminoacid zymogen. Exon 3 encodes the final part of the *gla* domain and a short hydrophobic *a*-helical stack of residues. Both exons 4 and 5 encode epidermal growth factor-like domains that are either involved in high affinity calcium binding [64] or play a role in binding to platelets and in the interaction of FIX with its co-factor, FVIIIa. The activation peptide is encoded by exon 6 and exons 7 and 8 encode the catalytic domain that includes the classical catalytic triad of His 221, Asp 269 and Ser 365. The plasma concentration of secreted FIX (0.3mg/dL) is ~50 times more than FVIII and has a better half-life (24 hrs) in circulation [15]. Catabolism of activated FIX may be *via* proteoglycans on the cell surface, which delivers factor IX to LRP, thus targeting FIX to the intracellular degradation pathway [65].

The distribution of different types of mutations resulting in hemophilia B shows that majority (~90% of ~1101 mutations) are single nucleotide variations identified throughout the *F9* gene from the promoter to the end of the coding region (HGMD®, Human Gene Mutation Database. http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F9). There is no *F9* gene mutation equivalent of the common *F8* inversions.

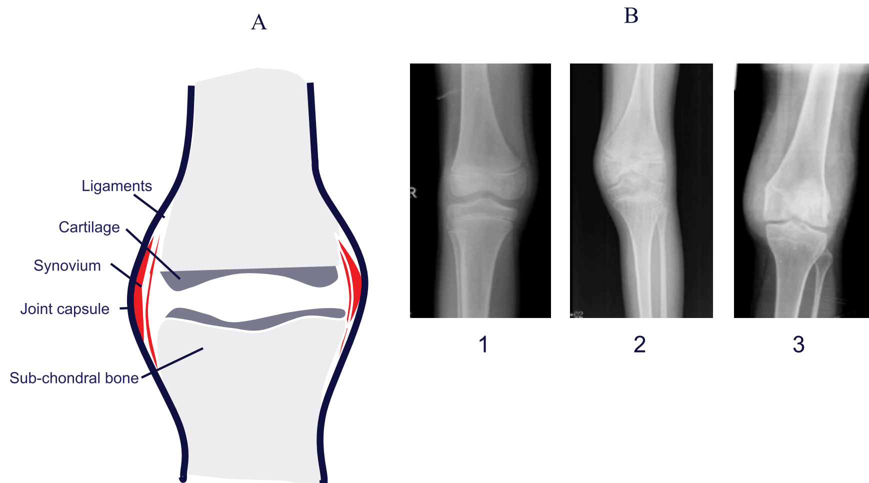

Figure 2: (**A**) **Joint architecture:** Synovial tissue: synthesizes synovial fluid, nourishes and lubricates the articular cartilage, enabling smooth movement of the joints. The synovial tissue has a synovial lining and the sublining. The synovial lining is composed of Macrophages (Type A synovial cells) and specialized fibroblasts (Type B synovial cells). The uptake of excess synovial fluid or breakdown products of cartilage is achieved by the synovial tissue. Articular cartilage is avascular, aneural, and depends mostly on the synovial fluid for its nutrition and maintenance. The main function of chondrocytes (sole population of cartilage) is the production and maintenance of extracellular matrix and balancing catabolic processes in the joint space. Blood induced joint damage is widely thought to happen by direct damage to the chondrocyte metabolism and integrity by components of the blood, while the indirect one is attributable to the inflammatory mediators and enzymes released by the inflamed synovium as a result of the blood in the joint cavity. **(B)** Radiographs of knee joints from normal individual (Panel 1), haemophilic patients with articular damage (Panel 2) and synovitis (Panel 3).

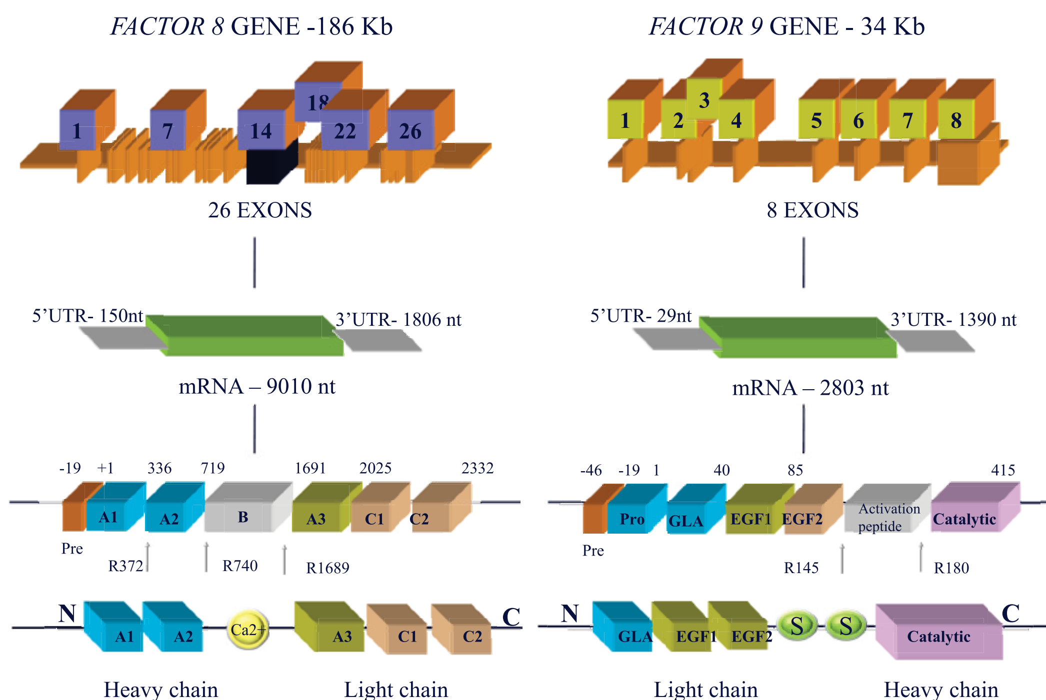

Figure 3: Factor 8 and factor 9 genes and proteins: *Factor 8* gene is 186 kilobases (kb) in length and encodes a messenger RNA of ~9kb. The newly synthesised factor VIII protein molecule is composed of a pre-sequence of 19 amino acids and a mature peptide of 2332 amino acids. The mature multi-domain factor VIII protein contains triplicated A domains, duplicated C domains and a single B domain. The arginine residues, which are the sites for proteolytic activation, are R372, R740, R1689. Activated factor VIII is a heterotrimer in which the dimeric N-terminal heavy chain is held together with the monomeric C-terminal light chain by a metal ion bridge (Ca^{2+}).
Factor 9 is 1/6th the size of *factor 8* gene, ~ 34 kb and encoding a transcript of ~1.4 kb. The mature factor IX protein consists of a pre- and pro-sequence and a mature peptide of 415 amino acids (total length, 461 amino acids). Activated factor IX has an N-terminal light chain and a C-terminal heavy chain held together by a disulphide bridge between cysteine resides 132 and 279. GLA, "*Gla*" domain, in which 12 glutamic acid residues undergo post-translational gamma-carboxylation by a vitamin K dependent carboxylase; EGF, epidermal growth factor-like domain; activation peptide released after proteolytic activation at arginine 145 and arginine 180; catalytic, the serine protease domain responsible for cleavage of factor X to Xa.

Of the mutations that appear more than once in the database, many are at 'CpG' dinucleotides that represent hypermutable sites in the genome, due to spontaneous deamination of 5-methylcytosine[66,67]. A significant proportion of mild hemophilia B (20-30%) is due a very small number of founder mutations [68,69]. Some mutations within the *F9* gene promoter region, such as the hemophilia B Leiden, that occur within constitutive transcription factor start sites but outside of hormonally regulated androgen response elements within *F9* promoter resolve spontaneously by adulthood [70] .

Diagnosis

Coagulation assays: Hemophilia is usually suspected when a typical soft tissue or musculoskeletal bleeding occurs either with or without family history of the disorder. This situation generally happens within the first year in patient with severe hemophilia and before 5 years in those with moderate disease [71,72]. Those with mild phenotype are often diagnosed only later in life, either post-traumatic or with pre-surgical screening procedures. Screening tests for clotting defects will show prolonged activated partial thromboplastin time (aPTT) but normal prothrombin time (PT), and normal thrombin time (TT) [73]. Further confirmation of the specific defect is by assays that measure the FVIII or FIX clotting activity (FVIII/FIX: C). Plasma FVIII or FIX inhibitor activity is assessed at regular intervals in patients who are receiving replacement therapy or if there is lack of response to it. This is also an aPTT based assay where the inhibitor titre is measured in Bethesda units. One Bethesda unit is the amount of inhibitor that will neutralize 50% of a given factor activity in normal plasma after a defined period of incubation [74]. While these clotting time based assays have been very useful for the diagnosis of hemophilia, they have not been able to discriminate the clinical heterogeneity of symptoms particularly in those with severe disease. It is possible that the tests of global hemostasis may be more useful in this regard but this is still a subject for research [75].

Genetic diagnosis: Before embarking on genetic diagnosis, it is imperative that detailed clinical evaluation and factor assays be available. There are two different approaches to the genetic evaluation of hemophilia. Analysis of single nucleotide polymorphism (SNP) or microsatellite variable number tandem repeat (VNTR) markers in the *F8* or *F9* gene to track the defective X chromosome in the family (linkage analysis) or identification of the disease causing mutation in the defective *F8* or *F9* gene (direct mutation detection) are employed [9,76].

Single nucleotide polymorphisms are commonly detected by PCR amplification of the target site followed by restriction fragment length polymorphism (RFLP) [9] whereas VNTRs are detected by conventional polyacrylamide gel electrophoresis [77] or by fluorescent PCR and capillary electrophoresis [78] (Figure 6). The key requirement for linkage analysis is the heterozygosity of the polymorphic marker in the mother of the index case. This requires a strategy for sequential analysis of different polymorphisms in *F8* or *F9* genes depending on heterozygosity rates in the population [78]. Although the principle on which linkage analysis is applied to hemophilia A and hemophilia B is similar, the severity of hemophilia A in the pedigree influences the diagnostic strategy employed. Many laboratories [79-81] in developing countries use linkage analysis following long PCR detection of two common mutations in the *F8* gene, the intron 1 or intron 22 inversions [59,82]. In inversion-negative cases and in patients with moderate or mild hemophilia A, several polymorphisms in the *F8* gene may be tracked [83].

Direct detection of disease causing mutation is informative in over 95% of families with hemophilia A and hemophilia B [1]. It is equally efficient and sensitive in detecting mutations in both familial and sporadic hemophilia, even in the absence of a proband. The strategy employed for point mutation screening includes amplification of the *F8* or *F9* gene (exonic and their flanking intronic regions, the 5'UTR and 3'UTR) by PCR followed by detection of mutations by various screening methods or/and DNA sequencing (Figure 7). For the *F9* gene, this is easier as it has only eight exons, the largest of which is less than 2 kb. In contrast, the large size and complexity of the *F8* gene necessitates amplifications of genomic DNA in over 30 fragments to cover the target regions [84]. However, with the declining cost of DNA sequencing reagents the adoption of direct nucleotide sequence analysis is becoming a viable option over mutation screening methods even for service laboratories [85]. Despite the varying choice of methods, it is clear that genetic diagnosis has significantly reduced the social and economic burden of hemophilia [76].

Treatment

There is no widely available curative treatment for hemophilia A and hemophilia B at present. The aim of current treatment strategies in hemophilia is to favorably alter the deranged hemostasis so that spontaneous bleeding is prevented and its resultant complications avoided [86]. This has been achieved so far by replacement of the deficient factor. Though the benefit of blood transfusion in hemophilia was established in the mid-19th century [87], it was not until a century later that the basis for this response was gradually understood [88]. Blood and plasma based treatment was limited by problems associated with availability, storage, accessibility and volume of infusion. With the discovery of cryoprecipitate [89], it became possible to achieve higher plasma levels without volume overload [90]. Once plasma could be fractionated to produce purified lyophilized clotting factor concentrates (CFC), prophylactic replacement of clotting factors became widely feasible. Continued manufacturing advances resulted in products of higher purity with fewer unwarranted proteins. Subsequently with the advent of recombinant FVIII and FIX [91-93], these products have become the standard of care, if accessible.

Two modes of replacement therapy evolved: one where replacement product is administered as and when bleeding occurred (episodic) and the other where it was administered to prevent bleeding (prophylaxis). Over the last four decades, considerable clinical experience in many countries with intensified replacement therapy has shown that the natural history of severe hemophilia can be significantly altered by

Mutation type	*Factor 8* gene % reported[1]	*Factor 9* gene % reported[2]
Missense /nonsense	60	64
Splicing	6.5	9
Regulatory	0.5	2.5
Small deletions	16.5	13
Small insertions	5	3.5
Small indels	1	1
Gross deletions/duplications	9	5.5
Gross insertions	1	0.5
Complex rearrangements	0.5	1

[1]HGMD®, Human Gene Mutation Database.http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F8 . This frequency distribution excludes the common inversion involving intron 1 and 22 seen in 45-50% of patients with severe hemophilia A.
[2]HGMD®, Human Gene Mutation Database.http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F9

Table 2: Frequency of disease-causing mutations reported in *factor 8* and *factor 9* genes.

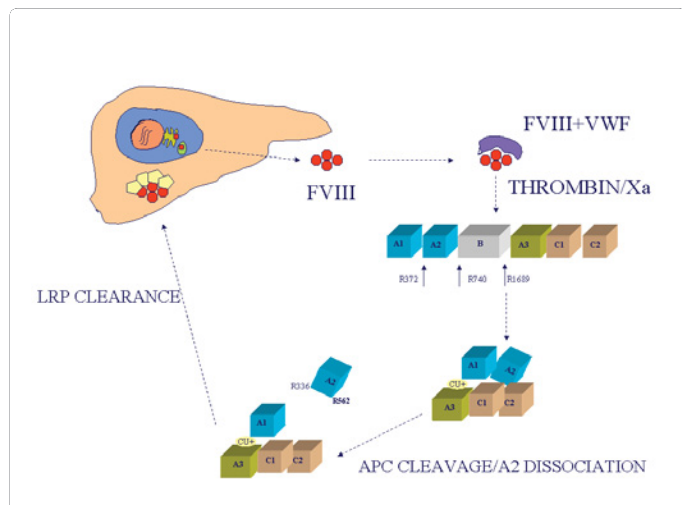

Figure 4: Inactivation and clearance of Factor VIII: Inactivation of FVIII comprises two distinct pathways: proteolytic degradation and spontaneous dissociation. Activated FVIII is intrinsically unstable, attributed to the weak interaction between the A2 domain and the metal ion-linked A1/A3-C1-C2 dimer [52] and therefore spontaneous dissociation occurs as the equilibrium is in favor of the inactive, dissociated state of FVIIIa at a physiological pH. Proteolytic degradation of FVIIIa involves cleavages in the heavy chain at positions 336 and 562 by various enzymes, such as FIXa, FXa, and activated protein C (APC) [51]. Cleavage at position 336 in FVIIIa releases a acidic sequence that interconnects the A1 and A2 domain. Because of this release, the A2 domain dissociates more rapidly from the FVIIIa heterotrimer. Arg562, which is part of the A2 domain sequence that comprises a FIXa interactive site, is exclusively cleaved by APC [147]. The relative contribution of each of these mechanisms to FVIII inactivation is not fully understood. FVIII catabolism is mediated by low-density lipoprotein receptor-related protein (LRP), a multiligand hepatic receptor, which belongs to low-density lipoprotein (LDL) receptor superfamily of endocytic receptors [53]. LRP-mediated clearance of FVIII from its complex with VWF is facilitated by cell-surface heparin sulphate proteoglycans (HSPGs), one of the major glycoprotein components of the extracellular matrix [148,149]. These HSPGs provide primary binding sites for FVIII, thus concentrating it on the cell surface and presenting it to LRP. Interaction of FVIII with LRP involves multiple, at least three, FVIII binding sites: within the A2 domain of the heavy chain and the C2 and A3 domains of the light chain [54].

both of these approaches though prophylaxis is clearly superior in preventing bleeds and preserving musculoskeletal function [94].

Prophylaxis: In prophylaxis, factor concentrates are administered regularly with the intention of preventing spontaneous hemarthrosis in patients with severe hemophilia. The concept that the prevention of bleeds was possible and desirable evolved in the late 1950s in Malmo, Sweden. It was supported by the clinical observation that patients with moderate hemophilia, with factor levels of >1%, had only occasional spontaneous bleeding and therefore maintained good joint integrity [95]. Patients who are treated with intensive prophylactic factor replacement can preserve normal musculoskeletal function and have a near normal quality of life. Subsequently, various groups have confirmed the benefit of regular prophylaxis in severe hemophilia (thrice a week or alternate days in hemophilia A and twice a week in hemophilia B at 25-40 IU/kg/dose) [96-99]. Prophylaxis generally begins by 1-2 years of age, by which time most severely affected children would have experienced their first joint bleed [100]. A recent randomized joint outcome study comparing episodic treatment with prophylaxis [101], showed a clear superiority of prophylaxis over episodic treatment even though patients receiving the latter approach had received doses of over 3000 IU/kg/year. Many questions remain unanswered regarding the dose and frequency of administration for prophylaxis and the dose required to treat musculo-skeletal or post-operative bleeds [102]. Most patients in developed countries are treated with doses of 25–40 IU/kg for such bleeds [103], while those in developing countries often receive lower doses of 10-25IU/kg[102]. Comparative studies of different doses for prophylaxis and treatment bleeds are very much needed. Similarly, factor replacement regimens for post-operative hemostasis vary widely in dose and duration. Although maintaining factor levels of ~80-100% is necessary during surgery, progressive reduction is possible during post-operative period over 7 to 14 days depending on the type of surgery [104]. This is another area in need of prospective studies.

Episodic therapy: Prophylaxis is out of reach for a majority (~80%) of patients with hemophilia, especially those in developing countries, due to the high cost and limited access to such factor concentrates [105]. Thus, most patients from these countries receive 'episodic' replacement of CFC for treatment of bleeds. However, this form of treatment is highly ineffective in preventing progressive joint damage. Retrospective data on episodic treatment over a wide range of doses of nearly 20 fold from 100 IU/kg/year to almost 2000 IU/kg/year, has shown very similar radiological joint scores by the time these patients reached about 20 years of age [102]. Even in the recent randomized joint outcome study [101], patients receiving episodic therapy who had used doses of over 3000 IU/kg/year, still had several joint bleeds every year. Bleeding episodes could be much worse at lower annual doses. It is therefore suggested that even when 1000-1500 IU/kg/year of CFC is available, patients could receive prophylaxis at lower doses (10-20 IU/kg two-three times a week). By doing this, it would be reasonable to expect the bleeding frequency to be significantly reduced in comparison to episodic treatment. There is some recent data to support this approach [106].

Adverse effects of treatment

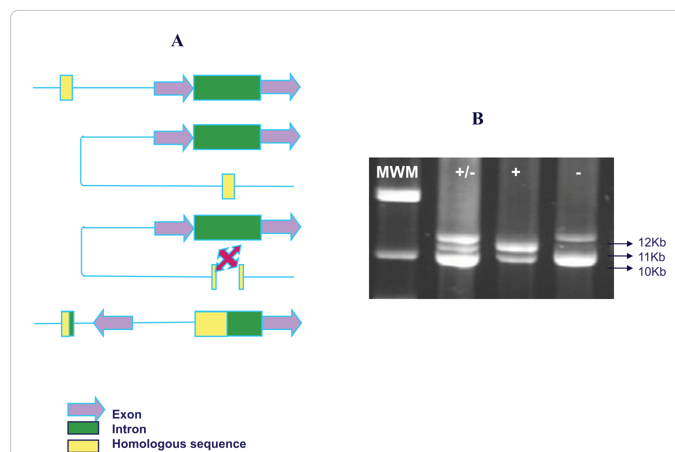

Figure 5: Common inversion involving intron 22 of factor 8 gene. A. The *F8* gene inversion arises from homologous recombination between int22h-1, a region within intron 22 of the *F8* gene, and one of two additional copies of the int22h-1 (a/b) region located approximately 500kb, 5' and telomeric to the *F8* gene. The int22h regions are approximately 9.5kb in length and have 99% homology with one another. Due to intra-chromosomal crossing over between the homologous sequences, an inversion of exons 1-22 away from exonic region 23-26 occurs and disrupts the factor VIII protein. This leads to severe hemophilia A in ~35% to 45% of all cases.
B. The intron 22 inversion is commonly detected by Long PCR, using two sets of primers specific for the F8 intragenic and extragenic copies and by the differential migration of amplicons during agarose gel electrophoresis. Thus a normal male (-) will exhibit a 10 and 12 Kb amplicon while inversion positive patient will exhibit 10 and 11 kb amplicons. A carrier female (+/-) can be identified by the presence of all the three PCR fragments.

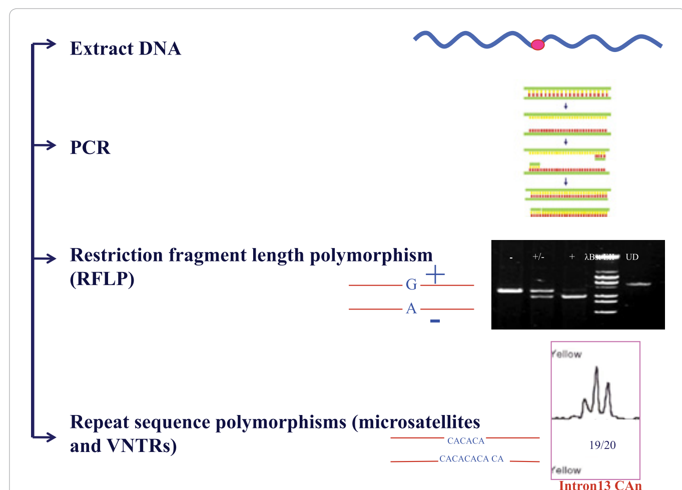

Figure 6: Linkage analysis: Following its isolation from peripheral blood, genomic DNA from patients and their parents are amplified for *factor 8* or *factor 9* gene intragenic or extragenic polymorphisms [*Factor 8* gene intron 7 G/A, intron 13 (CA)n, intron 18 BclI, intron 19 HindIII, intron 22 XbaI, intron 22 MspI, intron 22 (CA)n, intron 25 BglI site. *Factor 9* gene 5' MseI, intron 1 DdeI, intron 3 XmnI, intron 4 TaqI, intron 4 MspI, exon 6 MnlI, and 3' HhaI sites]. The amplicons are then screened for the bi-allelic polymorphic sites by either restriction fragment length polymorphism analysis (RFLP) or the multi-allelic sites by polyacrylamide or capillary electrophoresis. The resultant genotypes are used to identify the segregation of defective X chromosome in the family and interpret whether a proband is a carrier of hemophilia.

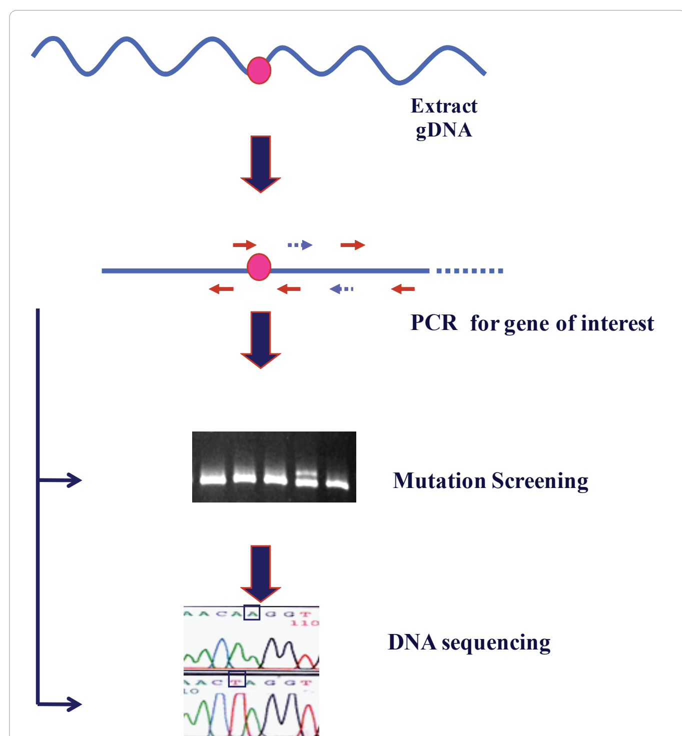

Figure 7: Direct mutation screening: Genomic DNA from patients and normal control are amplified for *factor 8* or *factor 9* gene coding and flanking intronic regions by a PCR. These amplicons are either screened by mutation screening methods to identify the PCR fragments that display heteroduplexes and sequenced to confirm the nature of nucleotide change. Alternatively, whole gene sequencing on all the PCR amplicons can be performed to identify the mutation.

Inhibitors of FVIII and FIX: The term 'inhibitor' refers to the development of allo-antibodies that neutralize the clotting factor activity. This is now the most significant challenge to factor replacement therapy. The incidence of inhibitors in patients with FIX deficiency is significantly less (3-5%) than those with FVIII deficiency (25- 30%) [107-109]. Inhibitors are more frequently encountered in persons with severe hemophilia compared to those with moderate or mild hemophilia. Any failure to respond to adequate CFC replacement therapy in a previously responsive patient is an indication to screen for an inhibitor. Patients with low titres of inhibitors (<5BU) can be treated with very high doses of the specific factor, if possible, to neutralize the inhibitor with excess factor activity and stop bleeding [110]. Patients with high titre inhibitors (>5BU) are generally treated with bypassing agents such as recombinant factor VIIa (rFVIIa) and activated prothrombin complex concentrates (APCC) such as the FEIBA™ [111,112]. Treatment with the latter may result in anaphylaxis and nephrotic syndromes in some patients with FIX inhibitors [113].

Eradication of inhibitors can be achieved by frequent administration of large doses (50-200IU/kg once a day) of the particular factor for several weeks to months (immune tolerance induction) [114]. Success is variable and optimal regimens for immune tolerance induction have not been established.

The development of inhibitors is confounded by several variables (Table 3). They include the severity of illness, age at the first infusion of replacement therapy [115]. Some retrospective data suggest that use of recombinant products may be associated with increased risk (36-39% Vs 20-33%) of developing inhibitors over plasma derived products [116]. However, there are limitations of such analysis. A major randomized study is currently on-going to address this issue (www.sippet.org). The risk of inhibitors in black patients is higher, almost twice that in white patients [117]. This may be related to mismatches in FVIII haplotypes of replaced recombinant concentrates [118].

The propensity for inhibitor development also has at least two genetic components, one of which relates to the type of clotting factor gene mutation and the other(s) likely involves elements of the immune system. In case of hemophilia A and hemophilia B, patients who carry a severe molecular defect (large deletions, inversions and nonsense mutations) that result in the complete absence of the coagulant protein have a higher propensity to develop inhibitors compared to those with missense or splice site mutations, where some residual FVIII/FIX antigen is present [109,119,120]. This is supported by the reported inhibitor prevalence of 21-88% in hemophilia A and 6-60% in hemophilia B patients with severe defects as opposed to <10% prevalence in patients with mild molecular defects [121]. The discordance for inhibitor development seen in patients or siblings with identical gene mutations suggests that other genetic factors play a modifier role [122]. Several polymorphisms in the genes encoding immunoregulatory cytokines and molecules such as human leukocyte antigen (HLA) class II, interleukin (IL)-10, cytotoxic T-lymphocyte antigen (CTLA)-4, tumor necrosis factor (TNF)-alpha and specific *F8* haplotypes have been shown to be associated with the development of inhibitors in patients with hemophilia A [118,123-126]. This remains an area of active research. Several large studies are ongoing results of which are eagerly awaited [127,128].

Transfusion transmitted infections: The emergence and transmission of HIV and hepatitis B and C through clotting factor products resulted in high morbidity and mortality of people with hemophilia in the 1980s [129,130]. These infections remain a risk for those people with hemophilia who continue to be treated with FFP and cryoprecipitate. However, this risk has been reduced as factor

Category	Determinants
Genetic factors	Type of FVIII/FIX mutation
	Functional polymorphisms in genes encoding cytokines, their receptors, immune-regulatory molecules that participate in antigen processing and T-cell and B-cell function.
Treatment-related factors	Age at first factor replacement
	Type of factor concentrates used and mode of administration
	Frequency and dose of administration
Environmental factors	Race and ethnicity of patient
	Challenges to Immune system (Infections, allergy etc.)

Table 3: Possible determinants of inhibitor development in patients with hemophilia.

concentrates are manufactured under current good manufacturing standards [131]. This is a result of the implementation of multiple risk mitigating steps, which include careful selection and screening of donors of source plasma, advances in sensitive diagnostic technologies for the detection of various pathogens [132] and multiple effective virucidal steps in the manufacturing process. However, further challenges remain from new and re-emerging infections, many of which are not amenable to current risk reduction measures. These include the non-lipid enveloped viruses and prions, for which diagnosis and elimination methods remain to be established [133,134].

Genetics of hemophilia and its translational impact

It is obvious that several aspects of hemophilia care have improved substantially over the last 5 decades. The availability of a large amount of mutation data in *F8* and *F9* genes (Table 2) has also helped in better understanding the biology of this disease. We now know that a majority of severe hemophilia A phenotypes occur due to an intra-chromosomal recombination between the original copies of intron 22/ intron 1 within the *F8* gene and the pseudocopies located telomeric to *F8* gene. Such inversions, together with other mutations (deletions, frameshifts, nonsense mutations) that significantly alter FVIII structure contribute a major risk factor for inhibitor development. Availability of robust techniques for genetic diagnosis of hemophilia has allowed families to make an informed choice for carrier detection and prenatal diagnosis [80,135]. In addition, the availability of mutation data has also had therapeutic impact in patients with hemophilia. An early phase clinical trial in which patients with nonsense mutations were administered gentamicin to override their ribosomal machinery is promising, although concerns regarding the toxicity of this approach remain [136].

Once the *F8* and *F9* genes were cloned [61,137] they were used for expression in recombinant systems. These studies contributed to the development of recombinant products and gene therapy. Kaufman *et al.* [50] demonstrated that limited thrombin mediated proteolytic cleavage that removes the B-domain is necessary for FVIII activation [50]. This finding subsequently paved the way for the successful clinical use of recombinant B domain-deleted (BDD) FVIII which is not only functional [138] but has also not shown an increased risk of inhibitor formation compared to products based on the full-length molecule [139]. Indeed, the limited size of the 4.3 kb BDD-FVIII has made it a preferred choice for gene delivery for hemophilia A as well [140]. The other major translational impact of genetics has been in bio-engineering of FVIII and FIX proteins which has significantly improved their activity and half-life [141]. Bio-engineered FVIII and FIX molecules with prolonged half-life are in clinical trials [141]. Other hemostatic agents such as fucoidans and aptamers are also being investigated in pre-clinical models and clinical trials [142,143]. The main challenges in this form of treatment will be to overcome limitations of frequent venous access, cost of novel replacement products and the development of inhibitors. Alternatively, gene-based [144] or gene-editing therapies [145] with targeted *in vivo* or *ex vivo* strategies, a subject which is dealt in great detail in other chapters of this issue, are also being developed. These may dramatically alter future therapeutic paradigms in the management of this condition. In particular, the promise of gene therapy as demonstrated recently in a clinical trial for hemophilia B [146], offers a realistic hope for cure from hemophilia. As novel and more effective treatments develop, it will also be important to ensure that steps are taken towards their equitable distribution and access around the world.

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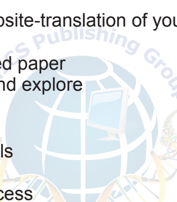

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