Expert Opinion

Peptides, Proteins & Antisense

Haemophilia therapies

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In the last few decades dramatic improvements in the management of haemophilia patients have occurred. Haemophilia has moved from a fatal or disabling disease to a hereditary disorder with available treatment and much better clinical outcomes. The safety of antihaemophilic factor concentrates has been dramatically improved and, in a multidisciplinary environment including haematologists, orthopaedic surgeons, paediatrics, infectiologists, specialised nurses and physiotherapists, complications related to haemophilia are now limited, markedly improving the quality of life of haemophiliacs. One can even think that the cure of haemophilia through gene therapy migth occur in the next decades. Keeping this ultimate aim in mind, efforts at present are mainly focused on bioengineered Factor VIII/Factor IX concentrates with increased efficacy or longer half-life or decreased immunogenicity. In addition, several preclinical and clinical studies are being carried out for optimising and individually tailoring the therapeutic regimens of antihaemophilic therapies using global haemostasis tests in combination with the routine coagulation assays.

Keywords: bypassing agents, FIX, FVIII, gene therapy, global haemostasis tests, haemophilia

1. Introduction

Haemophilia is a sex-linked genetic disorder resulting in a deficiency in Factor VIII (FVIII; haemophilia A) or Factor IX (FIX; haemophilia B) coagulant activity. In most patients, the plasma level of FVIII/FIX predicts the clinical severity of the disease. Severe haemophilia patients are subject to frequent intra-articular and intramuscular bleeds, and patients who are not on prophylaxis have an average of 20 – 30 episodes of spontaneous or trauma-related bleeding per year [1]. Treatment usually consists of replacing the missing coagulation factor from exogenous sources. The earliest descriptions of haemophilia were found in the Egyptian papyri and in the Talmud [2]. In the mid-nineteenth century it was recognised that transfusion therapy could treat bleeding in haemophilia patients [3]. In 1929 the first plasma substitution therapy for haemophilia was introduced [4] by precipitation of a globulin from plasma with low pH water. This globulin stopped the bleeding tendency in haemophiliacs more effectively than plasma. In 1939 Brinkhous [5] showed that haemophilia patients had a deficiency in this globulin. In 1952 Biggs et al. [6] introduced the term 'Christmas disease', which was later called haemophilia B by Cramer et al. [7]. Modern and effective management of haemophilia only became possible with the development of concentrated forms of plasma-derived coagulation factors in the 1970s. Since then, plasma-derived FVIII/FIX concentrates have undergone significant safety improvements. Unfortunately, during the 1980s many haemophiliacs were infected with blood-borne viruses, including hepatitis C and HIV, due to plasma-derived antihaemophilic factor concentrates that were capable of transmitting infectious diseases [8]. This led to dramatic improvements in viral inactivation methods and improved donor selection. These modifications to clotting factor products significantly reduced the threat of contamination with HIV, hepatitis B virus

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Table 1. Widely used plasma derived Factor VIII/Factor IX concentrates.

Plasma-derived factor concentrates	Manufacturer	Viral inactivation/removal
Factor VIII concentrates		
Factane	LFB	DPS + S/D + NF
Octanate	Octapharma	DPS + S/D + H
Monoclate P	ZLB Behring	DPS + P
Hemofil-M	Baxter	DPS + S/D
Emoclot DI	Kedrion	DPS + S/D + H
Fanhdi	Grifols	DPS + S/D + H
Intermediate purity FVIII concentrates		
Haemate P	Octapharma	DPS + P
Koate DVI	Talecris	DPS + S/D + H
Humate-P	ZLB Behring	DPS + P
Factor IX concentrates		
Betafact	LFB	DPS + S/D + NF
Octanine F	Octapharma	DPS + S/D + NF
Mononine	ZLB Behring	DPS + sodium thiocyanate + UF
Aimafix D.I.	Kedrion	DPS + S/D + NF
NOVIX	Grifols	DPS + S/D + NF

DPS: Donor plasma screening; H: Heat-treated; NF: Nanofiltration; P: Pasteurisation; S/D: Solvent/detergent; UF: Ultrafiltration.

(HBV) and hepatitis C virus (HCV); consequently, no infections have been reported since 1987. However, the risk still exists for parvovirus, hepatitis A virus (HAV) and emerging pathogens such as prions [9]. Recombinant FVIII/FIX concentrates, with very remote infectious risk, were developed in the late 1980s. In the last 50 years, obvious developments in haemophilia therapies and improvements in comprehensive haemophilia treatment centres have significantly improved the prognosis for haemophilia patients. This has transformed the status of haemophilia from a fatal disease to a bleeding disorder for which effective and safe therapies are readily available.

2. Plasma-derived Factor VIII/Factor IX concentrates

Efficacy, safety, cost and availability are the main criteria by which replacement therapy are evaluated and chosen. Plasma-derived factor concentrates are still used in most countries. Access to treatment in developed countries has improved the quality of life for patients by dramatically increasing their level of education and employability [10]. The safety of plasma-derived FVIII/FIX concentrates has been dramatically improved in the last 15 years. The procedures used to inactivate viruses include solvent/detergent treatment, heating, pasteurisation, chromatography techniques and nanofiltration. The solvent/detergent treatment is very effective at inactivating enveloped viruses, such as HIV, HBV

and HCV; however, it lacks efficacy against viruses without a lipid envelop, such as HAV or parvovirus B19. As a consequence, since 1997, more than one virucidal method has been used to inactivate non-enveloped viruses [11]. Nanofiltration consists of filtering protein solution through membranes with a pore size between 15 and 40 nm. It has been demonstrated that this method did not alter protein structure or function [12]. The screening of plasma samples using polymerase chain reaction testing was made obligatory in Europe in 1999 [11]. In addition, nucleic acid testing is employed for screening pooled plasma for HIV and HCV in the US [13]. Source plasma is systematically screened for HIV, HCV and HBV, and some manufacturers also screen for HAV and parvovirus B19. Furthermore, it is recommended that all haemophilia patients be vaccinated against hepatitis A and B, and it is noteworthy that no transmission of emerging infectious diseases, such as variant Creutzfeld-Jacob disease, has been reported in patients with haemophilia so far.

Plasma-derived concentrates are now quite safe; however, these safety measures are associated with a decreased yield of antihaemophilic factors extracted from plasma and are responsible for a subsequent increase in cost per unit. Low manufacturing yields are typically associated with high-purity products that exhibit a high specific activity. High-purity concentrates commonly have a higher solubility and there are fewer allergic reactions with these products, making them more suitable for home treatment. One advantage of less pure FVIII concentrates is that they contain von Willebrand factor

Table 2. Widely used recombinant Factor VIII/Factor IX concentrates.

Recombinant factor concentrates	Manufacturer	Viral inactivation	Comments
Factor VIII concentrates			
Advate	Baxter	S/D	- Full-length rFVIII - No vWF - Plasma/albumin-free - Formulated with sugar - No added animal/human-derived materials in cell culture, purification and formulation
Kogenate FS	Bayer	S/D	- Full-length rFVIII - No vWF - Formulated with sucrose - Albumin not added as excipient
Helixate FS	ZLB Behring	S/D	- Full-length rFVIII - No vWF - Formulated with sucrose - Albumin not added as excipient
ReFacto	Wyeth Pharmaceuticals	S/D	- B domain-deleted Factor VIII - No vWF - No albumin added in formulation - No added animal/human-derived materials in cell culture, purification and formulation
Factor IX concentrates			
BeneFIX	Wyeth Pharmaceuticals	NF	- No albumin added

NF: Nanofiltration; rF: Recombinant Factor; S/D: Solvent/detergent; vWF: von Willebrand Factor.

(vWF), and some studies have shown that the incidence of inhibitor development was lower in patients using FVIII concentrates containing vWF [14,15]. A list of widely used, commercially available (as of 2006) plasma-derived FVIII and FIX concentrates is presented in **Table 1**.

Unfortunately, in many developing countries where cryoprecipitate and fresh-frozen plasma are the only available treatments, a significant amount of transmission of blood-borne infectious agents still occurs. In addition, there is a risk for serious allergic reactions, such as transfusion-related acute lung injury due to cytotoxic antibodies contained in the infused plasma [16]. The wider prescription of recombinant factor concentrates in developed countries will probably force the manufacturers of plasma-derived concentrates to look for new markets in developing countries as prices become more competitive. This may allow for easier access to treatment for patients in the developing world [17].

3. Recombinant Factor VIII/Factor IX concentrates

The interest in developing recombinant FVIII/FIX concentrates was driven by the concept of safer therapies for haemophilia. The human *FVIII* gene was cloned in 1984 [18,19], and it was subsequently inserted in a plasmid vector and transfected to mammalian cells that express and secrete the FVIII protein. Mammalian cells are the only cells that have the capacity to perform the required post-translational glycosylation of the FVIII protein. Thus, the FVIII produced using Chinese hamster ovary and baby hamster kidney cell lines is very similar to human plasma-derived FVIII protein.

In the late 1980s, two pharmaceutical companies developed two preparations of full-length recombinant FVIII (rFVIII): Recombinate® (Baxter Healthcare) and Kogenate® (Bayer Healthcare) (**Table 2**). The main advantages of recombinant factor concentrates are their safety in terms of potential of viral transmission, as well as the fact that the manufacturing process of recombinant products does not rely on plasma supply. Several clinical studies demonstrated the excellent haemostatic effect and safety of recombinant clotting factors [20,21]. Recombinant factor concentrates used albumin in cell cultures and in the final product as a stabiliser. Indeed, high-purity rFVIII is unstable without albumin which allows FVIII concentrates to be stored for several months. Thus, the first generation of rFVIII products used animal- and human-derived components in the cell culture, with serum albumin added to the final formulation.

In the second generation of rFVIII concentrates, sucrose replaced albumin in the formulation, but animal- and human-derived ingredients were still added to cell cultures and during the protein purification phase [21]. Among the second-generation rFVIII concentrates, ReFacto® (Wyeth, Genetics Institute) contains a shortened FVIII protein;

specifically, it is a B domain-deleted molecule (**Table 2**) [22]. The B domain of FVIII does not contribute to the haemostatic activity of the FVIII protein [23], and in the absence of this B domain, rFVIII was found to be better secreted by Chinese hamster ovary cells. Haemostatic efficacy of the B domain-deleted rFVIII was demonstrated in on-demand and prophylactic treatments, as well as surgical situations [24]. However, some studies reported increased incidence of breakthrough bleeding with the B domain-deleted FVIII concentrate [25]. This could be explained, at least in part, by difficulties of laboratory monitoring of the drug rather than the inferiority of the product.

The hypothetical transmission of emerging pathogens that might be associated with the human- or animal-derived raw material has driven the development of third-generation rFVIII towards further improvements in virus safety. The first product of this new category, Advate rAHF-PFM (Baxter Healthcare), does not use any animal- or human-derived additives in any of the manufacturing steps (**Table 2**) [26].

The *FIX* gene was cloned in 1982 [27] and its nucleotide sequence was elucidated in 1984 [28]. However, genetic engineering of the recombinant FIX (rFIX) molecule has been difficult due to the complex post-translational modifications that are required for FIX activity [29]. Efficacy in previously treated patients was satisfactory, but the *in vivo* recovery was lower than that of plasma-derived FIX concentrates [30]. This can be explained, at least in part, by minor post-translational differences occurring in the rFIX protein. In haemophilia patients who have previously been treated with plasma-derived FVIII concentrates, rFVIII rarely triggers inhibitor development [20,31,32]. Conversely, the incidence of inhibitor development in previously untreated patients is estimated to be 15 – 30% [33,34]. At present, there is no formal proof that recombinant factors are more immunogenic than plasma-derived FVIII concentrates, although this question remains a subject of debate [35].

4. Prophylaxis or on-demand therapy?

Severe haemophilia patients (FVIII/FIX < 1 International Units [IU]/dl) suffer from repeated bleeding episodes that result in chronic painful joint disease and deformity known as haemophilic arthropathy [36]. The conventional treatment for bleeding in haemophilia patients is the replacement of the missing clotting protein. In addition to on-demand treatment, replacement therapy can be given at regular intervals in order to prevent life-threatening haemorrhages and musculo-articular bleeding events [37]. The rationale for prophylactic treatment is to maintain clotting factor activity levels > 1% and, therefore, to convert the bleeding phenotype of patients with severe haemophilia to a milder bleeding pattern similar to that of patients with moderate haemophilia [38,39]. Several studies have demonstrated that primary prophylactic therapy leads to better outcomes [40] in comparison with on-demand treatment strategies. However, the only form of haemophilia prophylaxis

that is able to prevent arthropathy is prophylaxis started at an early age (usually before the age of 2 years) either before or after the first joint bleed [41]. With respect to the optimal prophylactic therapy dosing regimen, there is no ideal single model. The 25-year Malmö experience indicates that treatment is most effective when administered in large doses (25 – 40 IU/kg) ≥ 3 times/week [42]. However, such an intensive treatment in young children may be very difficult to carry out from home. In many cases, the insertion of a central venous access device is required, which is associated with a significant risk of infectious or/and thrombotic complications [43,44]. In some countries, dose-escalated prophylaxis is being investigated [45]. These regimens begin with once-weekly administration of FVIII/FIX concentrate. When breakthrough bleeding occurs, infusion therapy is increased by either the frequency or administered dose. At present, there is no international recommendation on prophylactic therapy regimens [46], and each haemophilia comprehensive care centre decides the dose and the frequency of infusions according to the locally established criteria. Due to the high cost and limited availability of factor concentrates, dosing is an important issue in prophylaxis therapy. Recently, the Medical and Scientific Advisery Council of the National Hemophilia Foundation has made recommendations in the US [47,48]. According to these recommendations, prophylactic therapy should be instituted early in patients with haemophilia A using 25 – 50 FVIII U/kg three times/week or every other day. These recommendations are very similar to the Malmö prophylaxis protocol.

The difficulty of venous access in administering such an intensive treatment is one of the major problems of long-term prophylaxis therapy in young children. This treatment strategy requires experienced nurses, time and space. Infection is the most frequent complication of central venous access devices. Review articles reported that 50 – 83% of haemophiliacs with inhibitors can be expected to experience an infection when they are infused daily [49]. Several cases of central venous catheter (CVC)-related thrombosis have been described in haemophiliacs [50,51]. In a prospective cohort study of unselected severe haemophilia patients with long-term CVC, Journaycake *et al.* [52] showed the presence of catheter-related thrombosis in 53% of patients using contrast venography. A total of 33% had clinical symptoms related to their CVCs and 20% had no clinical signs. In all thrombotic cases, the CVC had been in place for a minimum of 4 years. In patients with CVC, a regular clinical surveillance (clinical signs of venous thrombosis and difficulties during factor injection) and the removal of catheters as soon as peripheral venipuncture can be performed should be encouraged to prevent complications. The transient character of haemostasis correction by factor concentrates could partly explain why thrombus formation takes longer than in other categories of patients. The risk of catheter-related venous thrombosis in haemophilia justifies both clinical and radiological surveillance. An annual X-ray of the position of the catheter tip is recommended [53].

Table 3. Factor VIII and Factor IX bypassing agents.

Bypassing agents	Manufacturer	Viral inactivation/removal
FEIBA VH	Baxter	Vapour heating (10 h 60°C 190 mbar, plus 1 h 80°C 375 mbar)
NovoSeven (Niastase in Canada)	NovoNordisk	- Affinity chromatography - Solvent/detergent (TNPB/polysorbate 80)

Another limit to a wider application of prophylaxis therapy is the high cost of this treatment strategy [54], although it is generally admitted that prophylaxis is the method of choice for treating severe haemophilia patients.

5. Development of inhibitors and management of bleeds in inhibitor patients

The development of inhibitors occurs in 10 – 15% of all patients with haemophilia in response to FVIII/FIX replacement therapy [55]. Inhibitors are more likely to develop in severe haemophilia patients and several variables appear to affect inhibitor development, including age, ethnicity, family history and mutations (associated most commonly with large deletions, nonsense point mutations and the inversion of intron 22). The median number of exposure days until inhibitors appear is typically ~ 10 days and very rarely after 200 exposure days [56]. The reason why inhibitors develop in some, but not all, haemophilia patients is related to several causes: some antibodies are directed against sites of the FVIII molecule that are not involved in its function; anti-FVIII antibodies are neutralised by secondary mechanisms, and B and T cells can be rendered anergic by intrinsic mechanisms [57]. Finally, not all haemophiliacs generate inhibitors, as their major histocompatibility complex phenotype is such that a cellular immune response against FVIII is not initiated [58]. Inhibitors are classified as low-titre if the level is < 5 Bethesda Units (BU) and high-titre if the level is ≥ 5 BU. Patients with low-titre and low-responding inhibitors can be treated with higher doses of FVIII/FIX concentrate to saturate existing antibodies and provide excessive FVIII/FIX for haemostasis. However, 70% of inhibitors are due to high-responding or anamnestic antibodies, which exhibit an important rise in titre (≥ 5 BU) within 5 – 6 days after exposure to FVIII [59]. For patients whose inhibitor titres exceed 5 BU, FVIII replacement therapy is ineffective. In these patients bypassing agents (e.g., prothrombin complex concentrates [PCCs], activated prothrombin complex concentrates [APCCs] or recombinant activated FVII [rFVIIa]) or immunoadsorption of antibody or porcine-derived FVIII concentrates represent effective treatment strategies for the treatment or prevention of haemorrhages.

5.1 Prothrombin complex concentrates and activated prothrombin complex concentrates

Although historical data reported no difference in efficacy between APCCs and PCCs, APCCs appear to be more effective than PCCs, and APCCs, at present, are used most widely in therapeutic practice [60,61]. At present, FEIBA® (Baxter Healthcare) is the only APCC still available on the market. FEIBA was licensed for the treatment of bleedings in inhibitor-developing haemophiliacs (**Table 3**). It contains activated Factor X, prothrombin (Factor II), FIX, Factor VII (FVII), protein C, thrombin, activated FVII (FVIIa) [62] and trace amounts of FVIII [63]. FEIBA has been reported to successfully stop ~ 80% of joint and soft tissue bleeds in patients with inhibitors [64]. Four major trials assessed the clinical efficacy and safety of FEIBA and showed significant improvement in bleeding control [65-68]. The largest trial on the use of FEIBA investigated 433 bleeding episodes, of which 81.3% demonstrated a good efficacy of the drug in control of the bleeding, with an excellent tolerance in 98.8% of cases [68]. However, an anamnestic response was noted in 31.5% of cases. FEIBA has also been shown to be effective in surgical situations [68]. Thrombotic events, including acute myocardial infarction, have been reported with FEIBA treatment. Due to the thrombotic risk, it has been recommended that the maximum daily dose of FEIBA should not exceed 200 U/kg [69]. Recently, the results of a 10-year compilation of thrombotic adverse events with FEIBA were published [70]. Ehrlich *et al.* [70] reported that the risk of thrombotic complications in patients receiving FEIBA was low. In addition, recognition of thrombotic risk factors and attention to dosage recommendations were found to be important for avoiding thrombotic adverse events. APCCs should be used with caution in patients with a personal history of vascular occlusive disease [71]. The concurrent use of APCCs with antifibrinolytics should also be avoided [63,71]. Other adverse events are mostly minor and include fever, chills, nausea and flushing in < 4% of cases, and chest pain and breathing discomfort in < 1% of patients [63]. FEIBA undergoes viral inactivation procedures and there has been no reported viral transmission due to the introduction of these safety procedures [72,73]. Another concern is the absence of a routine laboratory test for monitoring the efficacy and potential thrombogenicity of the APCCs. Recently, it has been shown that the thrombin generation test (TGT) and thromboelastography (TEG) could be of value for the monitoring of FEIBA therapy [74-76].

5.2 Recombinant activated Factor VII

Recombinant FVIIa (rFVIIa) (NovoSeven®, NovoNordisk, Bagsvaerd, Denmark) is structurally similar to human plasma FVIIa (**Table 3**). The mechanism of action of rFVIIa is complex and not completely understood. NovoSeven increases

formation of rFVIIa/tissue factor complexes, leading to improved generation of activated Factor X [77] and thrombin [78]. NovoSeven can bind to the surface of activated platelets in the absence of tissue factor and activates Factor X directly on platelet surfaces [79]. The binding of rFVIIa to activated platelets could explain why rFVIIa is supposedly only localised at the site of vascular injury [80]. Finally, rFVIIa enhances thrombin activatable fibrinolysis inhibitor (TAFI) activation, which inhibits early fibrinolysis and participates in the maintenance of the clot formed [81]. rFVIIa is effective at achieving haemostasis in haemophilia patients with an inhibitor. Elective surgery in haemophiliacs can safely be undertaken with rFVIIa therapy. A randomised controlled trial compared two doses of rFVIIa (35 and 90 µg/kg) in patients with haemophilia A or B with inhibitors who were undergoing minor or major elective surgical procedures, and showed that rFVIIa 90 µg/kg appeared to be effective and safe for minor and major procedures [82]. Similar efficacy results were also published from the compassionate-use programme [83,84]. A prospective study assessing the efficacy of rFVIIa in 103 surgical cases reported an excellent efficacy in 81% of major, 86% of minor and 92% of dental procedures [83]. Ingerslev [85] published data relating to 21 surgical procedures and indicated that good results could be achieved using rFVIIa for surgery. The best evidence available for the use of rFVIIa in dental surgery was reported in a series of seven patients [86]. Haemostasis was successful in six patients treated with rFVIIa 60 – 90 µg/kg every 3 – 4 h. It was insufficient in the seventh patient, probably due to the very low doses (45 µg/kg) used in this patient. Effective home treatment achieving haemostasis in most patients using two or three injections of NovoSeven was also reported by several groups [87-89]. Adverse events, including venous thromboembolism, myocardial infarction, disseminated intravascular coagulation and early recurrence of bleeding, were reported in 116 out of 140,000 rFVIIa administrations [90,91]. The licensed dose for NovoSeven is 90 µg/kg every 2 h. Frequent bolus injections are required because of the short half-life of the molecule (∼ 2.9 h) [92]. Recent studies evaluating the efficacy of a single mega-dose of rFVIIa (> 200 µg/kg) reported a significantly higher efficacy of the megadose with a lower consumption of the product [93-95]. In order to determine optimal doses, several groups have assessed continuous infusion of rFVIIa [96-98]. However, the results have been controversial, with a variable efficacy in different studies and no obvious correlation between infused doses and observed clinical efficacy. As for APCCs, laboratory monitoring of rFVIIa therapy (e.g., prothrombin time and FVIII coagulant activity) do not correlate with the clinical efficacy of NovoSeven. Recently, global haemostasis tests, such as TEG [76,99] and TGT [100], have been proposed for monitoring NovoSeven treatment.

5.3 Immunoadsorption of Factor VIII inhibitors

Immunoadsorption is usually used in patients who do not respond to conventional treatment. Immunoadsorption is used to remove inhibitors by passing the patient's plasma through columns containing protein A or sheep antihuman immunoglobulin, which bind and trap inhibitors [63]. Anti-FVIII/FIX inhibitors are usually of the IgG4 and IgG1 subclasses [101]. In contrast to anti-immunoglobulin sepharose columns, which bind all classes of immunoglobulin, protein A columns strongly bind IgG1, -2 and -4 [102]. The immunoadsorption method is particularly useful in patients undergoing elective surgery because the technique can reduce inhibitor titre to < 5 BU, making the use of high-dose FVIII/FIX replacement therapy possible [75]. It has also been shown that immunoadsorption is effective at lowering FVIII inhibitor titres, making patients more sensitive to treatment with porcine FVIII [103]. It is noteworthy that protein adsorption columns and porcine FVIII are only available in some countries. Immunoadsorption is considered safe as there has been no serious reported column-related side effects; however, the technique requires specialised equipment, an experienced team and an adapted venous access.

5.4 Immune tolerance induction and inhibitor eradication

The management of patients with inhibitors includes the treatment of bleeding episodes and the permanent eradication of the inhibitor by immune tolerance therapy (ITT). ITT is based on long-term, uninterrupted exposure to FVIII/FIX in order to 'tolerize' the immune system, allowing for later reintroduction of specific factor replacement [104]. Successful ITT is defined by achievement of an undetectable level of inhibitor associated with a normal FVIII recovery and half-life [105]. It has been shown that lower pre-ITT inhibitor (< 10 BU), historical inhibitor peak < 200 BU and maximal peak after ITT initiation correlate with successful outcomes in severe haemophilia A patients with high-titre inhibitors [106]. ITT is usually initiated using 50 – 200 U/kg daily or every other day depending on locally established protocols [107]. Common practice is to start with the same product that induced the inhibitory response. Unfortunately, ITT fails in 20 – 30% of patients and an inhibitor may become a long-standing problem, severely affecting health and quality of life [108]. There is also some evidence that plasma-derived FVIII concentrates comprising vWF could rescue ITT-resistant patients [109,110], but these data have not yet been corroborated by prospective randomised data. The concomitant use of immunosuppressive agents has not proven useful, although it was suggested [111].

Recently, selective B cell depletion using a humanised anti-CD20 monoclonal antibody has been reported. Case report data indicated a beneficial effect in patients with acquired haemophilia [112], and a few studies reported the potential usefulness of this drug in congenital haemophilia patients with inhibitors [113]. However, the number of successful case reports in congenital severe haemophilia is very limited and more information is required [114].

6. Bioengineering of improved Factor VIII/Factor IX molecules

Factor concentrates do have some limitations, including a relatively short half-life and the development of inhibitors. Bioengineering of rFVIII might improve FVIII biosynthesis and secretion, prolong half-life and decrease immunogenicity. For FVIII, strategies to improve the efficiency of expression have included modifications that increase mRNA expression, reduction of interactions with endoplasmic reticulum chaperones and, improvement of the efficiency of endoplasmic reticulum–Golgi transport [115]. Furthermore, these modifications can be combined in the same molecule [116]. The long-acting rFVIII strategy includes the addition of polyethylene glycol (PEG) polymers and polysialic acids or PEG-modified liposomes [117]. PEG polymers incorporate many water molecules, and PEGylation increases the size of the associated molecule above the limit for kidney filtration, which confers a longer circulating half-life [118]. Incorporation of PEGylated lipids onto liposomes leads to a reduced reticuloendothelial system clearance and prolonged half-life [119]. rFVIII molecules with increased duration of cofactor activity and with reduced *in vivo* clearance have been developed. FVIIIa is inactivated by activated protein C, activated Factor X and activated FIX. Genetically modified rFVIII molecules resistant to activated protein C inactivation and also resistant to A2 subunit dissociation were produced [120]. These proteins with prolonged activity may exhibit a higher haemostatic efficacy at lower plasma concentrations, which can allow a longer interval between FVIII infusions to maintain effective haemostasis in haemophilia patients. Catabolism of FVIII is also mediated by low-density lipoprotein-related receptor (LRP) and low-density lipoprotein receptor [121,122]. The FVIII A2 and A3 domains are essential for FVIII–LRP interactions. Site-directed mutagenesis of LRP binding sites induces a prolonged FVIII lifetime. Another challenge is finding ways to decrease the immunogenicity of FVIII in order to decrease inhibitor development in haemophilia patients who were undergoing FVIII replacement therapy. Recombinant hybrid human–porcine FVIII molecules have been constructed, and these hybrid molecules might have decreased antigenicity and immunogenicity [123,124]. The development of these new proteins promises to be useful in both factor replacement therapy and gene therapy protocols.

7. Gene therapy

Haemophilia is well suited to gene therapy because it is due to a single gene defect and even a small increase in FVIII activity in blood can significantly improve the bleeding phenotype [125]. The success of gene therapy depends on the development of safe, non-immunogenic vectors that can efficiently introduce the therapeutic gene into target cells. Sustained expression of FVIII and FIX has been achieved in preclinical studies using different gene transfer technologies and a wide range of target tissues. Subsequently, six different clinical trials were initiated, all of which resulted in very limited clinical efficacy (**Table 4**) [126]. The only significant adverse events reported in these trials have been related to host immune responses, emphasising that immunological barriers continue to represent the main obstacle to achieving successful gene transfer in patients [127]. For example, in a clinical trial of gene therapy for haemophilia B, one patient receiving the highest dose achieved ~ 10% of normal plasma FIX:C activity for some weeks with a subsequent decrease of FIX levels associated with a substantial elevation of transaminase levels. This could be explained by a destruction of transduced hepatic cells by cytotoxic T lymphocytes raised against the liver cells expressing viral capsid proteins [128]. Other concerns are the possibility of germline transmission [129], systemic inflammatory responses related to the use of high doses of adenoviral vectors [130]. A final concern is that insertional mutagenesis can result in cancer. Out of ten children, two developed T cell leukaemia after gene therapy for severe combined immunodeficiency using a retroviral vector [131]. It is probable that the nature of the immunological disease and the therapeutic gene both contributed to the development of leukaemia in these patients.

8. Expert opinion

In the last 30 years, haemophilia therapy has improved dramatically and complications of haemophilia have significantly decreased through complication prevention and prophylaxis therapy. It is unknown when effective and safe gene therapy for haemophilia will be available for patients in routine clinical practice. Furthermore, the authors believe that bioengineered recombinant factor concentrates with longer half-life, better potency and less immunogenicity will probably be available earlier than gene therapy. This can significantly improve the prophylaxis regimens with a greater interval between infusions and with a better overall compliance with prophylaxis therapy. In the meantime, we can try to optimise the performance of the existing treatment options. Recently, the results of several *in vitro* and *ex vivo* studies have been published on the potential usefulness of global haemostasis tests, such as TEG [76,132,133] and the TGT, in haemophilia patients [134]. TEG measures the viscoelastic changes associated with fibrin polymerisation. TEG enables a complete evaluation of the clot initiation, formation and stability using whole blood. The main advantage of TEG is its bedside capability to give a result within 30 minutes of taking into account the role of coagulation factors, inhibitors, platelet functions and also the fibrinolytic system. TEG has been shown to reflect the clinical efficacy of APCCs and rFVIIa in patients with haemophilia A with inhibitors [133]. Recently, Young *et al.* [76] showed the utility of TEG for the individualisation of bypass therapy in haemophilia patients

Table 4. Clinical gene therapy trials.

Clinical trial	Haemophilia A/B	Vector	Target organ	Subjects	FVIII/FIX expression	Success Rate	Side effects
Kay MA *et al.* *Nat. Genet.* (2000) **24**:257-261 [138]	FIX	AAV	Skeletal muscle	3	17 weeks	1 patient: FIX > 1 IU/dl 3 patients: modest reduction in Factor use	No significant side effect
Roth DA *et al.* *N. Engl. J. Med.* (2001) **344**:1735-1742 [139]	FVIII	Fibroblasts transfected by FVIII plasmid	Omentum	6	10 months	3 patients: FVIII > 1 IU/dl	Invasive procedure No significant side effect
Powell JS *et al.* *Blood* (2003) **102**:2038-2045 [140]	FVIII	Retroviral vector	Peripheral blood mononuclear cells	13	1 month	9 patients: FVIII > 1 IU/dl 5 patients: reduction in bleeding frequency	Transient positive PCR signal for vector in semen
Manno CS *et al.* *Nat. Med.* (2006) **12**:342-347 [141]	FIX	AAV	Liver	7 (3 doses tested)	8 weeks	2 patients at the highest dose tested: FIX > 1 IU/dl	Elevation of liver transaminases Vector DNA detectable in semen
Jiang H *et al. Mol. Ther.* (2006) **14**:452-455 [142]	FIX	AAV	Skeletal muscle	8	10 months	No patient with FIX > 1 IU/dl Persistance of FIX expression in skeletal muscle	No significant side effect
Lu DR *et al.* *Sci. China B* (1993) **36**:1342-1351 [143]	FIX	Retroviral vector	Skin fibroblast	2	6 months	1 patient: FIX > 1 IU/dl	No significant side effect

AAV: Adeno-associated virus; FIX: Factor IX; FVIII: Factor VIII; IU: International Units; PCR: Polymerase chain reaction.

with bleeding episodes. The TGT is a global haemostasis assay reflecting the overall function of the blood clotting system. Both platelet-poor and -rich plasma samples can be tested. In platelet-rich plasma, the test also reveals the role played by platelets. Measuring the thrombin generation by the 'old' subsampling method was heavy and expensive in terms of time and money. In the last 15 years, Hemker and Beguin [135] developed a technique in which a fluorescent substrate is added to clotting plasma and the course of thrombin formation is monitored. Recently, the authors' group has published the mean values of TGT parameters in severe, moderate and mild haemophiliacs [136]. In addition, the authors' *ex vivo* results, obtained 24 h after FVIII concentrate administration, showed that in patients presenting with similar plasmatic FVIIII levels, thrombin generation capacity may be significantly different. This suggests that in haemophiliacs, TGT could be useful for individually tailoring prophylactic regimens, as well as for adapting clotting factor infusions in surgical situations. The authors also showed that TGT may significantly impact the decision-making process of the most adapted therapy in the treatment of high-risk, severe haemophilia patients with inhibitor [75]. It has also been shown that the TGT could be used for the monitoring of FVIII/FIX bypassing agents for which there is, at present, no laboratory monitoring test [74,100,137]. Taken together, these preclinical and clinical results strongly suggest that global haemostasis tests may be a promising tool for a novel approach to the management of haemophilia based on 'individual tailoring' of therapies rather than the 'same regimen for all'.

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