Gene Therapy for Haemophilia

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Abstract

The most effective treatments currently available for haemophilia A and B (factor VIII or factor IX deficiency respectively) require lifelong, regular, frequent intravenous infusion of highly expensive replacement protein that has a short half-life. Factor levels in blood follow a saw tooth pattern which is seldom in the normal range and often falls low enough to allow breakthrough bleeding. Most haemophiliacs worldwide do not have access to this level of care or in many cases any treatment at all. In contrast, gene therapy offers the potential for cure by techniques that result in continuous endogenous expression of factor VIII or factor IX following transfer of a functional copy to replace the haemophilic patient's own defective gene. Haemophilia is a strong candidate for gene therapy for several reasons; a small increment in blood factor levels (≥2% of normal) significantly improves the bleeding tendency; response can be easily and regularly monitored with a validated assay; tight regulation of expression is not required. The first trial to provide clear evidence of efficiency after gene transfer in patients with haemophilia B was recently reported by an Anglo-American group[refs1,2]. A single peripheral vein infusion of adeno-associated virus (AAV) vector containing the factor IX (FIX) gene led to dose dependent increase in plasma FIX at therapeutic levels with no persistent ill effects. The patients continue to be monitored and no late toxicities have been observed thus far, whilst plasma levels of factor IX remain stable for up to 6 years with corresponding reduction or elimination of replacement factor use and bleeding episodes. In this review we discuss the data from that study and results that are emerging from many similar studies in both haemophilia A and B. Because no reports from the most recent studies have yet been published in peer

reviewed journals, we include information from presentations at meetings and company press releases with appropriate caveats as to the need for caution in their interpretation.

Introduction

The commonest severe inherited bleeding disorder in all ethnic groups worldwide is haemophilia A, followed by Haemophilia B. These are X-linked recessive disorders which result from mutations in the genes for blood clotting factor VIII (FVIII) in haemophilia A or factor IX (FIX) in haemophilia B. The incidence of haemophilia A in live male births is approximately 1 in 5,000, and of haemophilia B, 1 in 25,000. Bleeding tendency varies but correlates best with the residual circulating factor level, which in turn depends on the genotype of the mutation that prevents synthesis and/or interferes with function of the affected factor. If the residual factor level is 5% of normal or greater, subjects can be assigned to the mild category, in which spontaneous bleeding is absent and only occurs after significant trauma. In the next lower range, where residual factor level is under 5% but above 1%, patients are considered to have moderate haemophilia with a variable bleeding tendency; some in this group seldom have any bleeding, while others experience frequent bleeding after minor trauma. Up to half of patients with haemophilia A or B have factor levels <1% of normal.[1] These individuals have a severe bleeding tendency with frequent spontaneous musculoskeletal and soft tissue bleeding. A recent careful study of the haemophilic patient population at a large Dutch clinic[2] confirmed these correlations and the basic division into severe, moderate and mild, but added the insight that those mildly affected patients whose residual factor level is greater than 13% never experienced joint bleeding. Thus attaining a steady state factor level >13% could be considered a target for gene therapy. Amongst those patients who do bleed into their joints, the ankles are most commonly affected starting in early childhood, with knees and elbows affected later.

Repeated episodes of intra-articular bleeding cause severe, progressive, destructive arthropathy with deformity leading to loss of joint function and attendant disability.

In the absence of replacement therapy the life expectancy of a boy with severe haemophilia is about 10 years. This still applies in less developed countries. Even in developed countries until the 1960s, treatment of haemophilia was limited to infusion of fresh frozen plasma or concentrates derived from animal plasma with short lived efficacy due to antibody formation. In 1968 the first widely available human plasma derived concentrate for haemophilia A –cryoprecipitate, was introduced[3]. During the 1970s and 1980s many multi-donor factor concentrates were developed to improve the purity, potency, stability and convenience of administration of factor replacement therapy. But these developments, depending as they did on ever larger donor pools of often commercially sourced plasma, led directly to transmission of HIV. Almost a whole generation of haemophiliacs who were given the new products became HIV positive and died of AIDS before highly effective antiretroviral therapies were developed. During the period 1970 to 1986 every treated patient was also exposed to hepatitis C and up to 25 years later some are still succumbing to chronic liver failure resulting from continued infection. From 1986 onward heat treatment and then the solvent detergent method inactivated both HIV and Hepatitis C virus. Since then there have been no new cases of transmission of those lipid enveloped viruses. Transmission by blood products of other pathogens resistant to inactivation, such as parvovirus,[2] hepatitis A[3] and prions (variant Creutzfeldt-Jakob disease[4]) remain a major concern. Recombinant factor concentrates are of course free from blood borne infections, but their availability has been limited to the most developed countries by very high cost. With the expiry of patents on recombinant

factor VIII and IX, biosimilars and other variants with enhanced pharmacokinetic or other properties are entering the market, with potential for wider availability than hitherto.

In developed countries standard haemophilia care for severely affected patients now consists of home administered prophylaxis with safe concentrates intended to maintain factor level above 1% of normal. This is a compromise based on cost and practical considerations which reduces but does not eliminate bleeding. If started in early childhood after the first joint bleed, arthropathy can be largely prevented [6]. When continued throughout life, prophylaxis leads to near normalisation of life expectancy[7]. The relatively short half-life of FVIII and FIX in the circulation necessitates frequent intravenous administration of factor concentrates (at least 2-3 times a week) which is demanding and extremely expensive; annualised costs of prophylaxis for an adult equal or exceed £120,000. Even with prophylaxis, significant limitations remain as normal plasma clotting factor levels are not consistently restored; the short half-life of existing clotting factors results in troughs of circulating clotting factor associated with break-through bleeding. The saw tooth pattern of factor level mandates careful planning of peak activities such as sport, to coincide with peak levels attained only briefly after infusion of factor. New modified synthetic formulations of factor VIII and IX that are pegylated or fused to proteins with long half-life such as albumin or Fc**γ** have greatly improved the pharmacokinetic activity profile for factor IX but have been less impressive for factor VIII, due to the dominant role of Von Willebrand factor (VWF) in determining its half-life. In any case these products do not remove the problems of lifelong intravenous administration, break-through bleeding and ever mounting cost. The cumulative effect of lifelong administration of pegylated proteins are unknown, as is the potential of fusion proteins to induce immune response.[5] Two other

entirely novel approaches to normalising thrombin generation in haemophilia are undergoing extensive trials at the time of this writing. The first is a factor VIII mimic consisting of linked antibodies, one of which binds factor IXa and the other factor X (Emicizumab[ref]). Although restoring thrombin generation to a degree comparable to factor VIII level of about 15% in patients with or without inhibitory antibody, there is a major difference from wild type factor VIII. The mimic is under no control of its activity being permanently active throughout the circulation. In contrast native factor VIII has very strictly controlled activity in both time and place of action; it circulates as a procofactor tightly bound to its carrier VWF; it is activated only at sites of clot propagation; it has a very short half-life after activation; it is inactivated by the protein C pathway. The consequences of these differences have recently emerged in thrombotic events [ref] occurring in patients treated with Emicizumab and either FEIBA or Factor VIIa used as adjunctive therapy for breakthrough bleeding. The second alternative approach is to lower the natural antithrombin level with antisense RNA technology [ref]. Both approaches have shown efficacy in reducing the rate of bleeding, but their use may be limited by risk of thrombogenicity and both still require lifelong injections without restoring normal haemostasis.

Even set against this scenario of widening therapeutic choice, gene therapy offers a strikingly attractive potential for cure by means of the endogenous production of FVIII or FIX following transfer of a normal copy of the respective gene. The haemophilias were recognised in the 1980s as good candidates for gene therapy because all their clinical manifestations are due to lack of a single protein that circulates in minute amounts in the blood stream. Years of clinical experience and the natural experiment of moderate

haemophilia prove that a small increase to 1-2% in circulating levels of the deficient clotting factor significantly modifies the bleeding diathesis; so even a modest response to gene therapy can be effective. Regulation of transgene expression is unnecessary since a wide range of FIX or FVIII levels is without toxicity and effective at reducing bleeding. Animal models such as FVIII- and FIX-knockout mice[6,7,8] and dogs with haemophilia A or B[9,10], have facilitated extensive preclinical evaluation of gene therapy strategies. The efficiency of therapy can be assessed easily by measuring plasma levels of FVIII or FIX. The cDNA for the gene encoding FIX is small and adaptable to gene transfer in many viral systems. In addition its expression pathway is significantly less complex than that of FVIII and it is natively expressed at higher levels. Consequently, more gene transfer studies have focused on haemophilia B than haemophilia A, but this is rapidly changing as the technology evolves.

First clinical studies of gene therapy in haemophilia

The most efficient way to introduce therapeutic genes into target somatic cells, a process referred to as transduction, is to use adapted wild viruses as vectors, using the machinery they evolved for transferring their own DNA or RNA into host cells. Targeted cells can either be cultured for ex-vivo gene transfer, or within organs for in-vivo delivery of vector. A number of gene transfer vehicles have been developed based on viral vectors (see Tables 1 to 4). Of 9 Phase I clinical trials conducted in subjects with haemophilia using these vectors one is currently continuing. Early studies with non-viral, onco-retroviral and adenoviral vectors appeared safe but did not result in sustained transgene expression at therapeutic levels. [11-13;14] Recombinant adeno-associated viral vectors (AAV) currently

show the most promise for gene therapy of haemophilia. These vectors have the best safety profile among gene transfer vectors of viral origin, since wild type AAV has never been associated with human disease. Safety is further enhanced by the dependence of AAV on co-infection with helper virus (usually adenovirus or herpesvirus) for productive infection. Additionally, recombinant vectors based on AAV are entirely devoid of wild type viral coding sequences, thus reducing the potential for invoking cell-mediated immune response to foreign viral proteins. Two clinical gene therapy trials for haemophilia B have been performed with AAV vectors based on serotype 2, the first serotype to be isolated and fully characterised (Table 4).[20;21] The first study was a dose escalation phase I/II study entailing multiple intramuscular injections of AAV vector encoding the FIX gene. Vector administration was not associated with serious adverse events. However, sustained increase in plasma FIX levels of >1% was not observed in any of the seven subjects recruited to this study despite immunohistochemical evidence of FIX expression at the site of injection for over 10 years.[20]

In the second study AAV2 vector containing a liver-specific expression cassette was infused into the hepatic artery over 3 different doses ranging from 0.08 to 2 x 10^{12}vg/kg. In all patients, vector genomes were transiently detected in the semen though there was no evidence of germ line transmission. The low and intermediate vector doses were safe but did not result in a detectable increase in plasma FIX levels. The results in the two subjects treated at the high dose level (2x10^{12}vg/kg) were mixed. One had higher levels of neutralizing anti-AAV-2 antibodies prior to gene transfer which appeared to block successful transduction, resulting in lack of any transgenic FIX expression. In contrast, FIX levels increased to around 10% of normal levels in the other subject for 4 weeks after

vector administration and then unexpectedly declined to baseline values. This decline in transgenic protein coincided with a transient 10-fold rise in liver transaminases, which spontaneously returned to baseline values over the subsequent weeks, consistent with a self-limiting process. Further studies have led to the hypothesis that the decline in FIX expression and the liver toxicity were likely due to a capsid-specific cytotoxic T cells directed against the transduced hepatocytes following presentation of AAV2 capsid peptide in the context of MHC I molecules.[21]

Thus, both humoral and cell mediated immune responses have the potential to limit persistent expression of FIX following administration of AAV vectors in humans.

Current and on-going trials of gene therapy for haemophilia A and B

In what follows continuing clinical trials of gene therapy for haemophilia using AAV based vectors are presented and discussed. The pace of advance is now so rapid that data on recently opened trials is only available as meeting presentations and/or company news releases, not yet as peer reviewed publications. Exceptionally therefore we are using those sources of information to bring readers of this review the most current available information, with the caveat that further experience may change our expectations of the safety and efficacy of gene therapy in haemophilia and of the most favourable combination of expression cassette, vector and trial protocol to attain improved or even normal levels of factor IX or factor VIII.

The first long term success in a clinical trial of gene transfer in haemophilia

Building on earlier studies discussed above an approach for gene therapy of haemophilia B was developed using a codon optimised version of the human *FIX* (*hFIXco*) gene was

synthesised and cloned downstream of a compact synthetic liver-specific promoter (*LP1*) to enable packaging into self-complementary AAV vectors (scAAV), which have a packaging capacity of approximately 2.3kb.[23] Preclinical studies in mice and non-human primates (NHP) showed that scAAV vectors were more potent than comparable single stranded AAV (ssAAV) vectors, raising the possibility of achieving therapeutic levels of FIX using lower and potentially safer doses of vector.[23;24]

Another important aspect of this study was to use a vector pseudotyped with AAV serotype 8 capsid. This had the advantage over AAV2 vectors, of the remarkable tropism of AAV8 for efficient transduction of the liver following administration of the vector in the peripheral circulation.[24;25] Hence a simple non-invasive route of vector administration was used that is safer for patients with a bleeding diathesis. Additionally, the lower seroprevalence of AAV8 in humans (~25% compared with over 70% for AAV2[26]), enabled exclusion of fewer subjects with pre-existing humoral immunity to AAV8.

Six subjects with severe haemophilia B were enrolled to the initial phase of this study with two subjects recruited sequentially at one of three vector doses (low [$2x10^{11}$ vg/kg], intermediate [$6x10^{11}$ vg/kg], or high dose [$2x10^{12}$ vg/kg]) of scAAV2/8-LP1-hFIXco. Factor IX expression at 1-6% of normal was established in all six subjects with an initial follow-up of between 6-14 months following gene transfer. Asymptomatic, transient elevation of serum liver enzymes, probably a result of a cellular immune response to the AAV8 capsid, was observed in both subjects recruited to the high dose level between 7-10 weeks after gene transfer. Treatment of each with a short course of prednisolone led to rapid normalisation of liver enzymes and maintenance of FIX levels in the 2-4% range. Four of the 6 subjects, have been able to discontinue routine prophylaxis without suffering

spontaneous haemorrhage, even when they undertook activities that previously had provoked bleeds. The other two have increased the interval between FIX prophylaxes. This is consistent with the natural bleeding history in mild haemophilia patients (FIX levels of between 5-40%) where bleeding episodes generally only occur after trauma or surgery with very few or no spontaneous bleeds.[27]

Longer follow-up of these individuals shows that AAV mediated FIX expression remained relatively stable over a period of at least 6 years.[28]. One of the four subjects who discontinued prophylaxis has subsequently been commenced on a once a week prophylaxis regimen to avert trauma-related bleeding incurred in the course of his work as a geologist. The others remain off prophylaxis and free of spontaneous haemorrhage. The overall reduction in FIX usage in these 6 subjects over the duration of the study is estimated to be approximately 2.2 million units so far and the resulting financial savings that exceed £1.5M. Subsequently a further four subjects were recruited for treatment at the higher dose. Two of these subjects had no evidence of immune mediated liver inflammation and achieved a level of stable factor IX expression between 5 and 8%. Both have stopped prophylaxis and report no bleeding. One subject had a mild episode of immune hepatitis that responded promptly to steroids. His factor IX level has been maintained at 5% and he has no need for prophylaxis and does not experience spontaneous bleeding since gene transfer. The remaining subject experienced a more marked elevation of transaminase which despite responding to a course of oral steroid was accompanied by a fall in steady state factor IX to 2%. He has less bleeding than prior to gene transfer, is not using prophylaxis but has occasional trauma related bleeding episodes requiring substitution therapy [ref]. In an on-going extension of the trial, the vector

preparation has been further purified to remove empty capsids and the optimum dose is being explored in dose escalation to determine if the immune hepatitis can be abrogated whilst attaining a therapeutically favourable factor IX level (A. Davidoff pers. comm.).

Recent trials of gene transfer in haemophilia B

5 studies of similar vectors for transferring either wild type factor IX or the gain of function mutation known as Padua (L349R) have been initiated since the first reports of successful long term expression noted above were published. The results of these trials as presented in meetings and/or released in communications from commercial sponsors are summarised in table 5. Of note the two studies using the Padua mutant are consistent with expression of a similar amount of protein as in the earlier St Jude/UCL trials but with 5 to 8 fold enhanced activity. Thus levels ranging from 20% to 40% have been recorded in 6 subjects. Of further note 2 out 6 subjects so treated in the study carried out by Spark therapeutics have had immune mediated elevation of liver enzymes and were treated with a course of oral steroid.

AAV vectors and gene therapy for Haemophilia A

The limited packaging capacity of AAV vectors (4680 kb) and the poor expression profile of FVIII have hindered the use of these vectors for gene therapy of haemophilia A. Compared to other proteins of similar size, expression of FVIII is highly inefficient.[29] Bioengineering of the FVIII molecule has resulted in improvement of FVIII expression. For instance, deletion of the FVIII B-domain, which is not required for co-factor activity, resulted in a 17-fold increase in mRNA levels over full-length wild-type FVIII and a 30% increase in secreted protein.[30;31] This has led to the development of BDD-FVIII protein concentrate, which is now widely used clinically (Refacto; Pfizer). Pipe and colleagues

have shown that the inclusion of the proximal 226 amino-acid portion of the B-domain (FVIII-N6) that is rich in asparagine-linked oligosaccharides significantly increases expression over that achieved with BDD-FVIII.[32] This may be due to improved secretion of FVIII facilitated by the interaction of six N-linked glycosylation triplets within this region with the mannose-binding lectin, LMAN1, or a reduced tendency to evoke an unfolded protein response.[33] These six N-linked glycosylation consensus sequences (Asn-X-Thr/Ser) are highly conserved in B domains from different species suggesting that they play an important biological role.[34]

Another obstacle to AAV mediated gene transfer for haemophilia A gene therapy is the size of the FVIII coding sequence, which at 7.0 kb far exceeds the normal packaging capacity of AAV vectors. Packaging of large expression cassettes into AAV vectors has been reported but this is a highly inconsistent process resulting in low yields of vector particles with reduced infectivity.[35;36] AAV vectors encoding the canine BDD-FVIII variant that is around 4.4kb have yielded promising results but further evaluation of this approach using human BDD-FVIII is required. Other approaches include the co-administration of two AAV vectors separately encoding the FVIII heavy- and light-chains whose intracellular association in-vivo leads to the formation of a functional molecule. [37] An alternative two AAV vector approach exploits the tendency of these vectors to form head to tail concantamers. Therefore, by splitting the FVIII expression cassette such that one AAV vector contains a promoter and part of the coding sequence, as well as a splice donor site, whereas the other AAV vector contains the splice acceptor site and the remaining coding sequence. Following in-vivo head to tail concatemerisation a functional transcript is created that is capable of expressing full length FVIII protein. [38-42] These two AAV vector

approaches are however inefficient, cumbersome, expensive and not easily transferred to the clinic.

We have developed an AAV-based gene transfer approach that addresses both the size constrains and inefficient FVIII expression. Expression of human FVIII was improved 10-fold by re-organisation of the wild type cDNA of human FVIII according to the codon usage of highly expressed human genes.[23;43-45] Expression from B domain deleted codon optimised FVIII molecule was further enhanced by the inclusion of a 17 amino-acid peptide that contains the six N-linked glycosylation signals from the B domain required for efficient cellular processing. These changes have resulted in a novel 5.2kb AAV expression cassette (AAV-HLP-codop-hFVIII-V3) that is efficiently packaged into recombinant AAV vectors and is capable of mediating supraphysiological levels of FVIII expression in animal models over the same dose range of AAV8 that proved to be efficacious in subjects with haemophilia B.

Juxtaposition of novel amino acid sequences as has been done in our AAV-HLP-codop-hFVIII-V3 could lead to neo-antigenicity, thereby increasing the risk of provoking a neutralizing antibody response to the transgenic protein. This was also a concern when recombinant BDD-FVIII (ReFacto) was first introduced for use in man. ReFacto contains the “SQ” link of 14 amino acids (SFSQNPPVLKRHQR) between the A2 and A3 domains, generated by fusion of Ser743 in the N-terminus with Gln1638 in the C-terminus of the B-domain, creating a neo-antigenic site. However, despite extensive clinical use of ReFacto, an increase in frequency of neutralizing hFVIII antibodies in patients treated with this product has not been observed.[46-48] Additionally, antibodies to epitopes in the B-domain that are occasionally seen in patients with severe HA treated with hFVIII protein

concentrates are devoid of inhibitory activity because they bind to nonfunctional FVIII epitopes.[49]

Using a AAV5 containing the SQ linker codon optimised factor VIII expression cassette described above, in a study sponsored by Biomarin 9 subjects with severe haemophilia A have been treated at doses ranging from xxx to xxx vg/kg. Of seven treated at the highest dose 6 subjects now have factor VIII level ranging from 50% to 250% (table 6). All were treated with prophylactic steroid after elevated transaminases were noted in the first of the seven subjects treated, whose factor level is now 20%. Highly encouraged by this result a new cohort of patients has been recruited to be treated at an intermediate lower dose level of xxx vg/kg in order to find a dose that does not cause response above the normal upper limit of 150% as seen in two subjects treated at the high dose level.

Obstacles to wider use of AAV vector technology

A. Safety considerations

Thus far, the risk of liver toxicity accompanied by loss or reduction of transgene expression appear to be the most worrying toxicity associated with liver targeted delivery of AAV, as described before. However, this phenomenon can be readily controlled with a short course of prednisolone and appears to be self-limiting with no evidence of persistent hepatocellular damage. The precise pathophysiological basis for the hepatocellular toxicity observed in this study remains unclear, in part because it has not been possible to recapitulate this toxicity in animal models. Longer follow-up of some of the high dose subjects in our study shows that cessation of prednisolone is not followed by a late rise in liver enzymes or reduction in transgene expression, presumably because capsid antigen

has been degraded and cleared from the remaining transduced hepatocytes by this point. The vector preparation used in this study contained a large excess (~80%) of empty capsids which are fully assembled capsids that lack a functional vector genome.[50] These empty particles cannot mediate FIX expression but can serve as antigenic targets for capsid-specific cytotoxic T cells following transduction of hepatocytes.[51] It is therefore logical to assume that removal of these contaminating empty particles, may allow administration of the high vector dose without provoking hepatocellular toxicity or compromising the level of gene transfer. As noted above this hypothesis is being tested further with a clinical lot of scAAV2/8-LP1-hFIXco from which empty particles have been removed by CsCl density centrifugation. The other strategy for reducing vector dose and, therefore, vector-related toxicity currently being investigated in the clinic entails the use of a self-complementary AAV vectors encoding FIX Padua potentially allowing correction of the severe bleeding phenotype HB with a lower vector dose. [52 53]

As expected, all subjects in these trials develop long lasting AAV capsid-specific humoral immunity. Whilst the rise in anti-AAV IgG does not have direct clinical consequences, its persistence at high titres precludes subsequent successful gene transfer with vector of the same serotype, in the event that transgene expression should fall below therapeutic levels. However, it has been established that it is possible to achieve successful transduction in animals including nonhuman primates with pre-existing anti-AAV8 antibodies following administration of AAV vector pseudotyped with an alternate serotype.[24] Based on follow up data in subjects with HB it is likely that retreatment may not be required for periods that extend beyond 6 years.

Another potential problem of systemic administration of AAV is spread of vector particles to non-hepatic tissues including the gonads. Vector genomes were transiently detectable in the semen of all subjects recruited to the AAV2 and AAV8 haemophilia B clinical trials.[27;54;55] The lack of persistence of the vector genome in semen of haemophilia B patients is consistent with animal data that suggested that AAV can transduce adventitial cells present in semen but not germ cells.

The risk of insertional mutagenesis following AAV mediated gene transfer has been judged to be low because proviral DNA is maintained predominantly in an episomal form. This is consistent with the fact that wild type AAV infection in humans, though common, is not associated with oncogenesis. However, deep sequencing studies show that integration of the AAV genome can occur in the liver.[56;57] Additionally, an increased incidence of hepatocellular carcinoma (HCC) has been reported in the mucopolysaccharidoses type VII (MPSVII) mouse model following perinatal gene transfer of AAV potentially through integration and disruption of an imprinted region rich in miRNAs and snoRNAs on mouse chromosome 12.[58] Subsequent studies in other murine models have failed to recapitulate this finding and collectively the available data in mice as well as larger animal models suggest that AAV has a relatively low risk of tumourigenesis.[59]

B. Scale-up of vector production

Continued progression toward flexible, scalable production and purification methodologies is a required to support the commercialisation AAV bio-therapeutics. The most widely used method for the generation of AAV entails the transient transfection of adherent HEK 293 cells with plasmids encoding the necessary vector, helper and packaging genes. The

appeal of this method is the flexibility and speed, which are important assets during the initial stages of development. Not surprisingly, therefore, almost all AAV vector preparations administered to humans in the last 10 years have been prepared by transient transfection of adherent HEK 293 cells. However, this method is cumbersome and not suited for production of large quantities of clinical-grade vector required for Phase III/market authorisation trials of haemophilia gene therapy. Attention has recently shifted to transfection of suspension culture-adapted 293 cells because they are more amenable to scale-up than using adherent cells.[60] Another scalable method for production of AAV that has received a lot of attention is one based on baculovirus.[61] This method has recently been used to support market authorisation of gene therapy for lipoprotein lipase deficiency. Impurities commonly found in AAV vector preparations include host cell proteins, mammalian DNA and empty capsids which as described above can affect safety. Therefore, attention need to be paid to the downstream purification process which typically consists of column chromatography so that the purity of clinical grade AAV preparation can be improved without compromising scalability.

Affordability of gene therapy

The World Federation of Hemophilia estimates that 80% of haemophilia patients receive no or only sporadic treatment and are condemned to shortened lives of pain and disability. This is in large part because the cost of prophylactic treatment with factor concentrates is high and in excess of £120,000 for an adult per year. This is unaffordable by the majority of the World's hemophiliacs.[62] It is likely that gene therapy will command a high price, at least initially, in order to recoup the development cost. However, successful gene therapy

offers the advantage of continuous endogenous expression of clotting factor which will eliminate breakthrough bleeding and micro-haemorrhages thereby reducing comorbidities and the need for frequent medical interventions whilst improving quality of life, thus yielding significant savings for the health care system and society in general. Therefore, if appropriately managed gene therapy has the potential to be affordable when all such factors are considered.

Conclusion

The availability of convincing evidence of long-term expression of transgenic FIX at therapeutic levels resulting in amelioration of the bleeding diathesis following AAV mediated gene transfer is an important step to the eventual licensure of gene therapy for haemophilia. Whilst, several obstacles still remain, the current rate of progress in this field suggests that a licenced gene therapy product will be commercially available within the next decade. This will like change the treatment paradigm for patients with severe haemophilia and, in addition, facilitate the development of gene therapy for other disorders affecting the liver where the treatment options are limited or non-existent.

Table I: AAV Properties applicable to all current clinical trials in haemophilia A and B								
Packaging capacity	**Ease of production**	**Integration into host genome**	**Duration of expression**	**Transduction of post-mitotic cells**	**Pre-existing host immunity**	**Safety concerns**	**Germ-line transmission**	
4.6 kb	*Cumbersome*	*Infrequent*	*Long-term in post-mitotic cells*	*Efficient*	++	*Immune response to capsid*	*Not observed*	

Table 2. ***Summary of Phase I-II studies in haemophilia B***								
Sponsor	**Transgene**	**Vector**	**Inclusion criteria**	**Method of vector delivery**	**Safety**	**Expression (% of normal)**	**Current status**	**References**
Children's Hospital of Philadelphia	hFIX	AAV-2	Adults with severe HB	IM	No significant side effects	Transient < 1.6%	Closed	
Avigen, Alameda, CA	hFIX	AAV-2	Adults with severe HB	Bolus infusion into hepatic artery	Transient transaminitis at 3 weeks after gene transfer	Transient hFIX at 10% in 1 pt given 2 $x10^{12}$vg/kg	Closed	
St Jude	Self complementary AAV Codon optimised FIX	AAV-8	Adults with severe HB	Bolus peripheral vein infusion	Transient transaminitis at 7-10 weeks after gene transfer	Persistent (>4 years) dose dependent expression of FIX at between 1-6% of normal level in all subjects recruited	Open	
CHOP	Single stranded AAV Codon optimised FIX	AAV-8	Adults with severe HB	Bolus peripheral vein infusion		Two subjects recruited at $1x10^{12}$vg/kg. Persistent FIX at ~8% of normal level in one	Closed	
Baxalta	Self complementary AAV Codon optimised FIX containing the Padua mutation	AAV8	Adults with severe HB	Bolus peripheral vein infusion	Variable response with loss of activity with or without transaminitis	Expression up to 50% but not maintained.	Closed	
Spark	Single strand AAV Codon optimised FIX containing Padua mutation	Spk 100	Adults with sever HB	Bolus peripheral vein infusion	Transaminitis in 2 out of 6 patients	Expression range 20% to 40%	Open	
Uniqure								

Table 6: Biomarin Haemophilia A study. Vector AAV8 containing FVIIIsq codon optimised sequence. Ref 20

Week**	20	24	28	32	36	40	4
n***	7	7	7	6	7	6	2
Median Factor VIII Level**** (%)	**97**	**101**	**122**	**99**	**99**	**115**	**11 9**
Mean Factor VIII Level**** (%)	**118**	**130**	**124**	**122**	**115**	**127**	**11 9**
Range (high, low)	(12, 254)	(16, 227)	(15, 257)	(26, 316)	(31, 273)	(17, 264)	(105, 133)

Factor VIII Levels (%) in High Dose Patients* by Visit (N=7)
**All patients had severe hemophilia A Factor VIII equal to or less than 1% of blood clotting factor.*

***Weeks were windowed by +/- 2 weeks*

****For week 32, one patient had no Factor VIII reading, for week 40, one patient had not reached week 40 and for week 44, only 2 patients reached a week 44 reading*

*****Bolded numbers are in normal range of Factor VIII as defined by the World Federation of Hemophilia, http://www.wfh.org/en/page.aspx?pid=643 (link current as of Jan. 8, 2017). Factor VIII levels are determined by one-stage assay.*

Table 7: Summary of Factor VIII Level (%) of High Dose Patients at Most Recent Evaluation (N=7)

High- dose Subject #	FVIII level (%) at last update in July 2016	Most recent week of observation	FVIII level (%) at most recent observation
1	89	50	121
2	219	42	133
3	271	40	222
4	12	41	16
5	133	40	175
6	69	38	77
7	79	34	62

Table 8: Summary of Annualized Bleeding Rate (ABR) and FVIII Infusions of High Dose Patients Previously on Prophylaxis (N=6)

	Before BMN 270 Infusion**	**After BMN 270 Infusion*****
	Mean (median, SD)	**Mean (median, SD)**
Annualized Bleeding Rate* (bleeding episodes per subject per year)	16.3 (16.5, 15.7)	1.5 (0, 3.8)
Annualized FVIII Infusions* (infusions per subject per year)	136.7 (138.5, 22.4)	2.9 (0, 7.0)

** Rates were based on data from week 3 after BMN270 infusion through last follow-up visit*

***Obtained from medical records.*

****5 of 6 patients had 0 bleeds requiring Factor VIII infusions and 0 Factor VIII infusions from Week 3 after BMN 270 infusion.*

Table 9: Summary of ALT Levels in High Dose Patients at Most Recent Evaluation (N=7)

	ALT (U/L); (ULN = 43 (U/L))		
High-dose Subject#	**Peak ALT level**	**ALT Level at Most Recent Observations**	**ALT Level Status**
1	60	15	Normal
2	95	16	Normal
3	82	42	Normal
4	87	33	Normal
5	43	38	Normal
6	81	45	<1.1 ULN
7	66	27	Normal

All patients currently off steroids.

Table 10 Commercial gene therapy products in clinical development for hemophilia

	Company	Product	Vector	Therapeutic gene	Manufacturing platform	Year in which first patients dosed in phase 1/2 trial
Hemophilia B	Shire	BAX 335	AAV8	Padua mutant factor IX	HEK293 cells	2013
	Spark Therapeutics/ Pfizer	SPK-9001	Engineered AAV	Padua mutant factor IX	HEK293 cells	2015
	uniQure	AMT-060	AAV5	Wild-type factor IX	Baculovirus	2015
	Dimension Therapeutics	DTX101	AAVrh10	Wild-type factor IX	HEK293 cells	2016
	Sangamo Biosciences	SB-FIX	AAV6	Zinc-finger-nuclease-mediated integration of wild-type factor IX into the albumin locus in hepatocytes	Baculovirus	Expected 2016
	Freeline Therapeutics	FLT-180	Engineered AAV	Undisclosed	HEK293 cells	Expected 2017
	Bioverativ	Undisclosed	Lentivirus	Padua mutant factor IX	HEK293 cells	Expected 2018
Hemophilia A	BioMarin	BMN 270	AAV5	B-domain deleted factor VIII	Baculovirus	2015
	Spark Therapeutics	SPK-8011	Engineered AAV	B-domain deleted factor VIII	HEK293 cells	Expected 2016
	Dimension Therapeutics/ Bayer	DTX-201	Undisclosed	B-domain deleted factor VIII	HeLa cells	Expected 2017
	Shire	BAX-888	AAV8	B-domain deleted factor VIII	HEK293 cells	Expected 2017
	Sangamo Biosciences	SB-525	AAV6	B-domain deleted factor VIII	Baculovirus	Expected 2017

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