Advances in Gene Therapy for Haemophilia

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Abstract

Gene therapy provides hope for a cure for patients with haemophilia by establishing continuous endogenous expression of factor VIII or factor IX following transfer of a functional gene copy to replace the haemophilic patient's own defective gene. Haemophilia may be considered a 'low hanging fruit' for gene therapy because a small increment in blood factor levels (≥2% of normal) significantly improves the bleeding tendency from severe to moderate, eliminating most spontaneous bleeds. After decades of research, the first trial to provide clear evidence of efficiency after gene transfer in patients with haemophilia B using adeno-associated viral (AAV) vectors was reported by our group in 2011. This has been followed by unprecedented activity in this area with the commencement of 7 new early Phase trials involving over 55 patients with haemophilia A or haemophilia B. These studies have, in large part, generated promising clinical data that lay a strong foundation for gene therapy to move forward rapidly to market authorisation. In this review, we discuss the data from our studies and emerging results from other gene therapy trials in both haemophilia A and B.

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Introduction

Haemophilia A and B are X-linked recessive disorders resulting from mutations in the gene for blood clotting factor VIII (FVIII) or IX (FIX) respectively. The incidence of haemophilia A is approximately 1 in 5,000, and that of haemophilia B is 1in 25,000 live male births. Collectively they are amongst the most common inherited bleeding disorders in the World. Despite the genetic and biochemical differences, these disorders are indistinguishable clinically with the severity of bleeding symptoms varying according to the residual factor activity in a patient's plasma. Patients with a mild bleeding phenotype have baseline plasma factor levels in excess of 5% of normal and typically have few spontaneous bleeding episodes. However, they may have prolonged and life-threatening bleeding after trauma or surgery. A recent study of the haemophilic patient population at a large Dutch clinic [1] showed that those mildly affected patients whose residual factor level is between 5-13% may have a significant bleeding diathesis, whilst those with a level >13% rarely or never experienced joint bleeding. Over half of the patients with haemophilia A or B have factor levels of <1% of normal.[2] These individuals have a severe bleeding tendency with frequent spontaneous musculoskeletal and soft tissue bleeding. 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 complete loss of joint function and attendant disability.

In the absence of protein replacement therapy, the life expectancy of a boy with severe haemophilia is only about 10 years. This still applies in many less developed countries. Even in developed countries, until the 1960s, treatment of haemophilia was limited to infusion of fresh frozen plasma. In 1968 the first widely available 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 large donor pools of often commercially sourced plasma, resulted in widespread transmission of HIV and hepatitis C virus. Almost a whole generation of haemophiliacs who were given the new products became HIV positive and

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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,[3] hepatitis A[4] and prions (variant Creutzfeldt-Jakob disease[5]) 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 and production constraints. With the expiry of patents on recombinant factor VIII and IX, biosimilars and other variants with enhanced pharmacokinetic or other favourable 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 for patients with haemophilia B. 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 peaks and troughs of circulating clotting factor associated with break-through bleeding. The “saw tooth” pattern of factor level, high immediately after infusion, falling rapidly to near base line, mandates careful planning of physical activities such as sport, which people living without haemophilia can hardly imagine. 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 stability profile for factor IX but have been less impressive for factor VIII due to the

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dominant role of Von Willebrand factor 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 an immune response.[6] Two other entirely novel approaches to normalising haemostasis in haemophilia A patients are undergoing extensive trials. The first is a synthetic factor VIII mimic consisting of linked antibodies, one of which binds factor IXa and the other factor X (Emicizumab)[7]. Although restoring haemostasis 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, whereas native factor VIII has very strictly controlled activity in both time and site of action. It circulates as a procofactor tightly bound to a carrier, it is activated only at sites of clot propagation and it has a very short half-life after activation. The consequences of these differences have recently emerged in thrombotic events occurring in patients treated with Emicizumab and another clotting agent.[8] The second alternative approach is to lower the natural antithrombin level with antisense RNA technology[9], which will also work in patients with haemophilia B. 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.

Rationale for gene therapy for haemophilia

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 experience of patients with 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

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wide range of FIX or FVIII levels is without toxicity and effective at reducing bleeding. Animal models such as FVIII- and FIX-knockout mice[10],[11],[12] and dogs with haemophilia A or B[13],[14], have facilitated extensive preclinical evaluation of gene therapy strategies. The efficiency of therapy can be assessed easily just 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 normally 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.

Previous gene therapy trials in haemophilia

Ten phase I clinical trials have been conducted in subjects with haemophilia using a variety of different approaches. The first study involved ex-vivo gene transfer of DNA encoding a B-domain–deleted factor VIII gene (BDD-hFVIII) into autologous fibroblasts prior to their implantation into the omentum of subjects with severe haemophilia A. This procedure was well tolerated with no major side effects but resulted in poor efficacy in part due to the silencing of the transgene by DNA methylation. [15, 16] Onco-retroviral vector-mediated gene transfer of the FIX gene into ex-vivo expanded autologous fibroblasts prior to their implantation into skin of patients with severe haemophilia B was similarly safe but ineffective with no long-term maintenance of expression.[17] In-vivo, systemic, administration of onco-retroviral vectors encoding the BDD-hFVIII gene with vector doses as high as 9×10^8 transduction units/kg was well tolerated but plasma FVIII activity of ~1% of normal was only transiently detected in 6 of 13 subjects. Systemic administration of "gutless" adenoviral vectors, encoding full length FVIII gene resulted in a transient increase in FVIII activity to ~3%. However, this study was closed early because of elevation of liver enzymes associated with thrombocytopenia.[18]

Attention then moved onto recombinant adeno-associated viral vectors (AAV) because of their favourable safety profile and an ability to mediate long term expression of transgene following transduction of post-mitotic tissues such as the liver or muscle (Table 1).[19, 20] 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

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serious adverse events but sustained increase in plasma FIX at levels >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.[19]

In the second study, an AAV2 vector containing a liver-specific expression cassette was infused into the hepatic artery. In one subject treated at the high dose level ($2x10^{12}$vg/kg) FIX levels increased to around 10% of normal levels at 4 weeks after vector administration and then unexpectedly declined to baseline values. This decline 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 cell attack directed against the transduced hepatocytes following presentation of AAV2 capsid peptide in the context of MHC I molecules.[20]

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

Building on the studies discussed above, an approach for gene therapy of haemophilia B was developed using a codon optimised version of the human *FIX* (*hFIXco*) gene under the control of a compact synthetic liver-specific promoter (*LP1*) packaged into self-complementary AAV vectors (scAAV).[21] 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.[21, 22]

An important aspect of this study was to use a vector pseudotyped with AAV serotype 8 capsid. This had the advantage over AAV2 vectors used previously of a lower seroprevalence rate in humans of ~25% compared to >60% with AAV2 [23], thus enabling exclusion of fewer subjects with pre-existing humoral immunity from participating in the clinical trials. Another advantage was the high tropism of AAV8 towards hepatocytes enabling the vector to be delivered into the peripheral circulation, unlike AAV2 which had to be delivered directly into the hepatic artery.

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],

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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, perhaps 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 tendency 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.[24]

Longer follow-up of these individuals shows that AAV mediated FIX expression has remained relatively stable over a period of at least 6 years.[25]. One of the four subjects who discontinued prophylaxis has subsequently commenced a once a week prophylaxis regimen to avert trauma-related bleeding that might be 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 several million units so far and a resulting financial savings that exceed £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. 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.

New haemophilia B gene transfer trials

The pace of advance in AAV gene therapy for haemophilia in the last 5 years has been so rapid that data from most new trials is only available from meeting presentations and/or company news releases. Exceptionally, therefore, we are using those sources of information to bring readers of this review the most current available information, with the understanding that further experience may change our expectations of the safety and efficacy of gene therapy in haemophilia (Table 1). Of note, the two studies using the Padua mutant are consistent with expression of a similar amount of FIX antigen as in the earlier St Jude/UCL trials but with 5 to 10 fold enhanced activity. Thus, FIX levels ranging from 18% to 80% have been observed in 10 subjects in the study sponsored by Spark Therapeutics following a single administration of a relatively low dose of $5x10^{11}$vg/kg. Of note however is that 2 out 10 subjects had elevation of liver enzymes requiring that they treated with a course of oral steroid. Nevertheless, these studies show for the first time that it is possible to "normalise" FIX levels following a single administration of AAV vectors. This is something that was felt to be desirable but unachievable just 10 years ago. Not surprisingly, therefore, the average bleed rate in these patients dropped to 0.4 compared to 11.1 prior to gene therapy.

Despite stable expression for >7 years, the concern with AAV gene therapy approaches described above is that the AAV genome, which is retained in the liver in an episomal form, will be lost over time with turn-over of transduced hepatocyte. This raises the possibility of FIX expression falling below the therapeutic threshold. To overcome this hypothetical obstacle, Sangamo Therapeutics are using AAV vectors to deliver zinc finger nuclease (ZFN) and a promoterless FIX gene in an in-vivo genome editing approach that targets the albumin gene locus. The ability to permanently integrate the FIX gene in this targeted fashion provides an opportunity for life-long stability of expression that will be particularly appealing when targeting children. A Phase I/II clinical trial evaluating this approach in adults with severe haemophilia B is open for enrolment. A major limitation of

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this approach is the need for two AAV vectors, one encoding the ZFN and the other carrying the FIX cDNA.

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.[26] 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.[27, 28] 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.[29] 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.[30] 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.[31]

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.[32, 33] 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. [34] An alternative two AAV vector approach exploits the tendency of these vectors to form head to tail concatamers. Therefore, by splitting the FVIII expression cassette such that one AAV vector

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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. [35-39] 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.[21, 40-42] 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.[43-45] 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.[46]

15 patients with severe haemophilia have been recently been treated with an AAV5 vector containing the SQ linker codon optimised factor VIII expression cassette described above,

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in a Biomarin sponsored Phase I/II trial. Of these seven received a dose of $6x10^{13}$ vg/kg and an additional six were infused at a lower dose of $4x10^{13}$ vg/kg. The other two patients in the study received lower doses ($6x10^{12}$vg/kg and $2x10^{13}$vg/kg) as part of dose escalation but did not achieve therapeutic efficacy. At one year after dosing, FVIII levels in the $6x10^{13}$vg/kg cohort were between 20-218% of normal (Table 2). In 3 of the 6 patients in the $4x10^{13}$ vg/kg dose level FVIII levels are reported to be in the normal range, whilst in the remaining 3 patients FVIII levels are in the mild range. All were treated with prophylactic steroids after elevated transaminases were noted in the first subject treated at the $6x10^{13}$vg/kg dose level.

Preliminary data from Spark Therapeutic's SPK-8011 Haemophilia A gene therapy trial in which the first cohort received $5x10^{11}$vg/kg of AAV vector pseudotyped with a hybrid capsid show stable expression at 11% one patient, whilst the second patient achieved a 14% level with follow-up extending beyond 12 weeks. There were no toxicities (including inhibitor formation) and neither patient required treatment with steroids as there was no transaminitis. The investigators have dosed a 3rd patient at the next dose level of $1x10^{12}$vg/kg.

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 in some patients appear to be the most worrying toxicity associated with liver targeted delivery of AAV. Corticosteroids appear to reduce hepatocellular damage in some patients in the haemophilia B trials. In the haemophilia A studies corticosteroids appear to have questionable value in limiting the transaminitis. The precise pathophysiological basis for the hepatocellular toxicity remains unclear, in part because it has not been possible to recapitulate this toxicity in animal models.

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.

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However, some studies report effective gene transfer with AAV5 vector in individuals with detectable anti-AAV5 antibodies.

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.[24, 47, 48] Recent studies indicate that proviral DNA can be detected in mononuclear cells and semen of some participants for as long as a year.

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.[49, 50] 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.[51] 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.[52]

B. Scale-up of vector production

Continued progression toward flexible, scalable production and purification methodologies is now underway 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.[53] Another scalable method for production of AAV that has received much attention is one based on baculovirus.[54] This method was used to support market authorisation of gene therapy for lipoprotein lipase deficiency, but there are concerns that AAV particles made using the baculovirus method have a lower potency. Two other viable scalable methods consist of the adenovirus infection of Rep/Cap integrated cell lines and recombinant HSV helper vectors that create an all-in-one infectious system. [55,56] 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 needs 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

Haemophilia is a ~$10B market opportunity, dominated by factor replacement therapy which are effective but expensive and highly invasive. This area has remained largely unchanged over the past 20 years, other than the introduction of extended half-life factor concentrates, which allow for modestly longer intervals between infusion times. In contrast, a single administration, gene therapy is highly effective with no long lasting safety concerns enabling many patients to enjoy life without fear of bleeding. It is likely that gene therapy will command a high price, at least initially, in order to recoup the development cost. 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. It is therefore highly likely that gene therapy will not reach this disadvantaged population unless the cost of goods comes down significantly through innovations that improve vector yield and or improve manufacturing efficiency. 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. These factors will need to be considered when evaluating the health economics of gene therapy for these conditions.

Conclusion

The availability of convincing evidence of long-term expression of transgenic FVIII and FIX at therapeutic levels resulting in amelioration of the bleeding diathesis following AAV mediated gene transfer is an important step towards the eventual licensure of gene therapy for haemophilia. Whilst several obstacles remain, the current rate of progress in this field suggests that a licenced gene therapy product will be commercially available within the next 5 years. This will likely 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.

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Reference List

1. den Uijl IE, Fischer K, Van Der Bom JG et al. Analysis of low frequency bleeding data: the association of joint bleeds according to baseline FVIII activity levels. Haemophilia 2011;17:41-44.

2. Nathwani AC, Tuddenham EG. Epidemiology of coagulation disorders. Baillieres Clin Haematol 1992;5:383-439.

3. Saldanha J, Minor P. Detection of human parvovirus B19 DNA in plasma pools and blood products derived from these pools: implications for efficiency and consistency of removal of B19 DNA during manufacture. Br J Haematol 1996;93:714-719.

4. Lawlor E, Graham S, Davidson E et al. Hepatitis A transmission by factor IX concentrates [see comments]. Vox Sang 1996;71:126-128.

5. Baxter T, Black D, Birks D. New-variant Creutzfeldt-Jakob disease and treatment of haemophilia [letter; comment] [see comments]. Lancet 1998;351:600-601.

6. Pipe SW. The hope and reality of long-acting hemophilia products. Am J Hematol 2012;87 Suppl 1:S33-S39.

7. Shima M, Hanabusa H, Taki M et al. Factor VIII-Mimetic Function of Humanized Bispecific Antibody in Hemophilia A. N Engl J Med 2016;374:2044-2053.

8. Oldenburg J, Mahlangu JN, Kim B et al. Emicizumab Prophylaxis in Hemophilia A with Inhibitors. N Engl J Med 2017.

9. Sehgal A, Barros S, Ivanciu L et al. An RNAi therapeutic targeting antithrombin to rebalance the coagulation system and promote hemostasis in hemophilia. Nat Med 2015;21:492-497.

10. Kundu RK, Sangiorgi F, Wu LY et al. Targeted inactivation of the coagulation factor IX gene causes hemophilia B in mice. Blood 1998;92:168-174.

11. Bi L, Lawler AM, Antonarakis SE et al. Targeted disruption of the mouse factor VIII

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gene produces a model of haemophilia A [letter]. Nat Genet 1995;10:119-121.

12. Wang L, Zoppe M, Hackeng TM et al. A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci U S A 1997;94:11563-11566.

13. Giles AR, Tinlin S, Hoogendoorn H et al. Development of factor VIII:C antibodies in dogs with hemophilia A. Blood 1984;63:451-456.

14. Evans JP, Brinkhous KM, Brayer GD et al. Canine hemophilia B resulting from a point mutation with unusual consequences. Proc Natl Acad Sci U S A 1989;86:10095-10099.

15. Roth DA, Tawa NE, Jr., O'Brien JM et al. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. N Engl J Med 2001;344:1735-1742.

16. Roth DA, Tawa NE, Jr., Proper J et al. Implantation of non-viral ex vivo genetically modified autologous dermal fibroblasts that express B-domain deleted human factor VIII in 12 severe hemophilia A study subjects. Blood 2002;100:116a.

17. Qiu X, Lu D, Zhou J et al. Implantation of autologous skin fibroblast genetically modified to secrete clotting factor IX partially corrects the hemorrhagic tendencies in two hemophilia B patients. Chin Med J (Engl) 1996;109:832-839.

18. Mannucci PM. Ham-Wasserman Lecture : Hemophilia and Related Bleeding Disorders: A Story of Dismay and Success. Hematology 2002;2002:1-9.

19. Manno CS, Chew AJ, Hutchison S et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 2003;101:2963-2972.

20. High KA, Manno CS, Sabatino DE et al. Immune responses to AAV and to factor IX in a Phase I study of AAV-mediated liver-directed gene transfer for hemophilia B. Blood 2003;102:154a-155a.

21. Nathwani AC, Gray JT, Ng CY et al. Self complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. Blood 2006;107:2653-2661.

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22. Nathwani AC, Gray JT, McIntosh J et al. Safe and efficient transduction of the liver after peripheral vein infusion of self complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. Blood 2007;109:1414-1421.

23. Gao GP, Alvira MR, Wang L et al. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci U S A 2002;99:11854-11859.

24. Nathwani AC, Tuddenham EG, Rangarajan S et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med 2011;365:2357-2365.

25. Nathwani AC, Rosales C, McIntosh J et al. Long-term Safety and Efficacy Following Systemic Administration of a Self-complementary AAV Vector Encoding Human FIX Pseudotyped With Serotype 5 and 8 Capsid Proteins. Mol Ther 2011;19:876-885.

26. Miao HZ, Sirachainan N, Palmer L et al. Bioengineering of coagulation factor VIII for improved secretion. Blood 2004;103:3412-3419.

27. Pittman DD, Alderman EM, Tomkinson KN et al. Biochemical, immunological, and in vivo functional characterization of B-domain-deleted factor VIII. Blood 1993;81:2925-2935.

28. Kaufman RJ, Pipe SW, Tagliavacca L et al. Biosynthesis, assembly and secretion of coagulation factor VIII. Blood Coagul Fibrinolysis 1997;8 Suppl 2:S3-14.

29. Cerullo V, Seiler MP, Mane V et al. Correction of murine hemophilia A and immunological differences of factor VIII variants delivered by helper-dependent adenoviral vectors. Mol Ther 2007;15:2080-2087.

30. Malhotra JD, Miao H, Zhang K et al. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proc Natl Acad Sci U S A 2008;105:18525-18530.

31. Davidson CJ, Hirt RP, Lal K et al. Molecular evolution of the vertebrate blood coagulation network. Thromb Haemost 2003;89:420-428.

32. Allocca M, Doria M, Petrillo M et al. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. J Clin Invest

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2008;118:1955-1964.

33. Lai Y, Yue Y, Duan D. Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. Mol Ther 2010;18:75-79.

34. Burton M, Nakai H, Colosi P et al. Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. Proc Natl Acad Sci U S A 1999;96:12725-12730.

35. Jiang H, Pierce GF, Ozelo MC et al. Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. Mol Ther 2006;14:452-455.

36. Chao H, Mao L, Bruce AT et al. Sustained expression of human factor VIII in mice using a parvovirus- based vector. Blood 2000;95:1594-1599.

37. Chao H, Sun L, Bruce A et al. Expression of human factor VIII by splicing between dimerized AAV vectors. Mol Ther 2002;5:716-722.

38. Chen L, Zhu F, Li J et al. The enhancing effects of the light chain on heavy chain secretion in split delivery of factor VIII gene. Mol Ther 2007;15:1856-1862.

39. Chen L, Lu H, Wang J et al. Enhanced factor VIII heavy chain for gene therapy of hemophilia A. Mol Ther 2009;17:417-424.

40. Ward NJ, Buckley SM, Waddington SN et al. Codon optimization of human factor VIII cDNAs leads to high-level expression. Blood 2011;117:798-807.

41. Radcliffe PA, Sion CJ, Wilkes FJ et al. Analysis of factor VIII mediated suppression of lentiviral vector titres. Gene Ther 2008;15:289-297.

42. McIntosh J, Lenting PJ, Rosales C et al. Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. Blood 2013;121:3335-3344.

43. Gringeri A, Tagliaferri A, Tagariello G et al. Efficacy and inhibitor development in previously treated patients with haemophilia A switched to a B domain-deleted

recombinant factor VIII. Br J Haematol 2004;126:398-404.

44. Lusher JM, Lee CA, Kessler CM et al. The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. Haemophilia 2003;9:38-49.

45. Pollmann H, Externest D, Ganser A et al. Efficacy, safety and tolerability of recombinant factor VIII (REFACTO) in patients with haemophilia A: interim data from a postmarketing surveillance study in Germany and Austria. Haemophilia 2007;13:131-143.

46. Lavigne-Lissalde G, Lacroix-Desmazes S, Wootla B et al. Molecular characterization of human B domain-specific anti-factor VIII monoclonal antibodies generated in transgenic mice. Thromb Haemost 2007;98:138-147.

47. Manno CS, Arruda VR, Pierce GF et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 2006;12:342-347.

48. Arruda VR, Fields PA, Milner R et al. Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. Mol Ther 2001;4:586-592.

49. Nowrouzi A, Penaud-Budloo M, Kaeppel C et al. Integration frequency and intermolecular recombination of rAAV vectors in non-human primate skeletal muscle and liver. Mol Ther 2012;20:1177-1186.

50. Li H, Malani N, Hamilton SR et al. Assessing the potential for AAV vector genotoxicity in a murine model. Blood 2011;117:3311-3319.

51. Donsante A, Miller DG, Li Y et al. AAV vector integration sites in mouse hepatocellular carcinoma. Science 2007;317:477.

52. Kay MA. AAV vectors and tumorigenicity. Nat Biotechnol 2007;25:1111-1113.

53. Grieger JC, Samulski RJ. Adeno-associated virus vectorology, manufacturing, and clinical applications. Methods Enzymol 2012;507:229-254.

54. Cecchini S, Negrete A, Kotin RM. Toward exascale production of recombinant adeno-associated virus for gene transfer applications. Gene Ther 2008;15:823-830.

55. Martin J, Frederick A, Luo Y et al. Generation and characterization of adeno-associated virus producer cell lines for research and preclinical vector production. Hum Gene Ther Methods 2013;24:253-269.

56. Conway JE, Rhys CM, Zolotukhin I et al. High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap. Gene Ther 1999;6:986-993.

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Table 1: Haemophilia B gene therapy with AAV vectors

Sponsor	Transgene	Vector	Method of vector delivery	Expression (% of normal) Toxicity	Current status
Avigen and CHOP	Wild type FIX	AAV2	IM	Transient < 1.6% No significant side effects	Closed
Avigen and CHOP	Wild type FIX	AAV2	Bolus infusion into hepatic artery	Transient hFIX at 12% in 1 patient and 4% in 2nd patient at 2 x10^{12}vg/kg Transient transaminitis at 3 weeks after gene transfer in 2 out 7 patients	Closed
St Jude/UCL	Codon optimised	AAV8	Bolus peripheral	Persistent (>6 years) dose dependent expression of FIX at between 1-6% of	Closed

	FIX		vein infusion	normal level in all subjects recruited Transient transaminitis at 6-10 weeks after gene transfer in 4 out of 10 patients	
Shire (Baxalta; BAX 335)	Codon optimised FIX containing the Padua mutation	AAV8	Bolus peripheral vein infusion	Persistent (>2 years) expression of FIX at 25% in 1 out of 7 patients recruited Transient transaminitis at 6-10 weeks after gene transfer in 2 out of 7 patients	Closed
uniQure (AMT-060)	Codon optimised FIX	AAV5	Bolus peripheral vein infusion	Persistent (>1 years) expression of FIX at 3-7% in 9 out of 10 patients recruited Transient transaminitis at 6-10 weeks after gene transfer in 3 out of 10	Open

				patients	
Spark Therapeutics (SPK-9001)	Codon optimised FIX containing the Padua mutation	AAV- SPK-100	Bolus peripheral vein infusion	Persistent (~>12 weeks) expression of FIX at average of 33% in 10 patients recruited Transient transaminitis at 4-8 weeks after gene transfer in 2 out of 10 patients	Open
Dimension Therapeutics (DTX101)	Codon optimised FIX	AAVrh10	Bolus peripheral vein infusion	Persistent (~1 years) expression of FIX at 3-8% in 6 out of 6 patients recruited Transient transaminitis at 6-10 weeks after gene transfer in 5 out of 6 patients	Closed
Sangamo Bioscience	Codon optimised	AAV6/Zinc-finger mediated	Enrolling patients		

(SB-FIX)	FIX	targeted integration into the albumin locus in hepatocytes	

Table 2: Haemophilia A gene therapy with AAV vectors

Sponsor	Transgene	Vector	Method of vector delivery	Expression (% of normal) Toxicity	Current status
BioMarin (BMN 270)	Codon optimised BDD-FVIII	AAV5	Bolus peripheral vein infusion	Persistent (>20 weeks) expression of FVIII 20-218% 13/15 patients recruited Transient transaminitis starting at 4 weeks after gene transfer in 13/15 patients	Open
Spark Therapeutics (SPK-8011)	BDD-FVIII	Hybrid capsid	Bolus peripheral vein infusion	Persistent (>12 weeks) expression of FVIII at 5x1012vg/kg between11-14% 2/2 patients	Open

				No transaminitis	
UCL/St Jude (GO-8)	Codon optimised FVIII; B domain replaced with V3 peptide	AAV8	Bolus peripheral vein infusion	Enrolling patients	Open
Dimension Therapeutics/Bayer (DTX-201)	BDD-FVIII	? AAVRh10	Expected to open in 2018		
Shire (SHP654)	BDD-FVIII	AAV8			
Sangamo Bioscience/Pfizer	BDD-FVIII	AAV6			