

## **Haemophilia, the journey in search of a cure. 1960 - 2020**

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### ***Summary:***

The single most important step on the path to our modern understanding of blood coagulation and haemophilia in the 20<sup>th</sup> century was taken by British Pathologist Robert Gwyn Macfarlane with his 1964 publication “An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier”. He and Rosemary Biggs had previously discovered factor IX in 1952 as the factor deficient in Haemophilia B. In 1973 Arthur Bloom defined the distinct role of factor VIII and von Willebrand factor in Haemophilia A and von Willebrand’s disease respectively. This was followed by the heroic efforts of the Tuddenham group towards the purification of factor VIII to homogeneity in 1982 leading to the cloning of the Factor VIII gene in 1984, which in turn enabled development of safe recombinant factor concentrates for patients with haemophilia. Brownlee cloned the factor IX gene in 1982 at the Sir William Dunn Institute of Pathology in Oxford. Next came the first successful trial of gene therapy for haemophilia B in 2011 by the Nathwani group at UCL, which has fuelled the current quest for a functional cure of Haemophilia A and B. The UK, has therefore, contributed significantly to key scientific and clinical advances in haemostasis over the last 60 years.

### ***Introduction:***

#### ***ET writes:***

My introduction to haemophilia was as a trainee pathologist at Liverpool Royal Infirmary in 1969, where one of my tasks was to make up cryoprecipitate to treat men with haemophilia A. One patient failed to respond, and my subsequent investigations established that he had developed an inhibitory antibody neutralising factor VIII activity. This together with the privilege to work under David Weatherall, then Reader in Medicine convinced me to pursue a career in Haematology and specifically Haemostasis. The British Haemostasis community was going through a renaissance following the landmark description of the cascade theory of blood coagulation by Robert Macfarlane, director of the Medical Research Council Blood Coagulation unit in Oxford, where many early advances in coagulation were made.(Macfarlane 1964) This included the description of Haemophilia B by Rosemary Brigg in his group, then known as Christmas disease named after their first known patient with this disease, Stephen Christmas.(Biggs et al. 1952) With David Weatherall’s recommendation I was appointed to a lectureship at the Welsh College of Medicine in the department of Haematology where Arthur Bloom had set up a haemophilia Centre focused on research into Haemophilia A and Von Willebrand disease. Arthur Bloom was one of the first to propose that factor VIII and von Willebrand factor (then known as factor VIII related antigen) were indeed separate molecular entities.(Bloom 1980) After 4 years in Cardiff I was hooked on factor VIII and moved to Connecticut, USA to work with Leon Hoyer, who was working on purification of FVIII. Using FVIII binding antibodies immobilised on Sepharose gel, I defined a process for separating FVIII from VWF antigen but in amounts that were too small to allow full

biochemical characterisation. After two years in Connecticut I moved to the Haemophilia centre at Royal Free Hospital to succeed Katharine Dormandy as Centre Director. Here, together with Frances Rotblat, Don O'Brien and Alison Goodall we generated the monoclonal antibodies that were key to the final purification of FVIII. Using 5 kg batches of cryoprecipitate in a dustbin sized stainless steel container and a Polyelectrolyte column developed by Sarah Middleton at Speywood we generated highly active FVIII at over 4000U per ml with no VWF.(Rotblat et al. 1983) With this preparation in hand, we established a collaboration with Genentech in San Francisco, at that time a relatively small Biotech, to use the protein sequence to find the FVIII gene, which we successfully achieved in 1984 and was described by John Maddox, then the Editor of Nature, as a technical feat without parallel.(Vehar et al. 1984. Wood et al. 1984) Next arose an opportunity to set up a Medical Research Council funded Haemostasis research group at Northwick Park Clinical Sciences Centre, which included studentship for haematology trainees to undertake PhD projects in molecular haemostasis. One of those who took up a PhD studentship was Amit Nathwani.

***ACN writes:***

Haematology appealed to me because it offered an array of stimulating subspecialties ranging from haemato-oncology to monogenetic disorders such as sickle cell disease, thalassaemia, haemophilia, and prothrombotic disorders; a special mix of clinical and laboratory medicine. John Goldman and Lucio Luzzatto at the Royal Postgraduate Medical School steered me towards an academic career, which started with a PhD with the MRC Haemostasis Research Group led by ET, where I spent three years under his and John McVey's supervision working on functional analysis of the human tissue factor gene promoter in human umbilical vein endothelial cells (HUVECs). This project entailed the transfection of reporter gene into HUVECs, which is when I started to wonder about the potential of gene transfer for treatment of monogenetic disorders through the introduction of a normal copy of the mutated gene into target cells. With guidance and support from David Weatherall, David Linch and Sally Davies, I secured a Wellcome Trust, Advanced Training Fellowship to pursue a gene therapy approach for sickle cell disease, using adeno-associated viruses (AAV) under the supervision of Dr Arthur Nienhuis, at St Jude Children's Research Hospital, Memphis, Tennessee USA. AAV is a small (25-nm), non-pathogenic single-stranded DNA parvovirus with an excellent safety profile and an ability to transduce a wide range of human tissues. Within 6 months, I realised that AAV vectors based on serotype 2, the serotype to be first isolated and characterised, were not suited for gene therapy of sickle cell disease. This is because the AAV genome is maintained predominantly in an episomal format following gene transfer into haematopoietic stem cells, our target for sickle gene therapy, and is rapidly jettisoned from these cells as they undergo division. (Nathwani et al. 2000).

Searching for inspiration, I recalled how my PhD examiner, Professor George Brownlee, who had isolated the gene for factor IX, suggested that I work on gene therapy for haemophilia B. I, therefore, switched tack, though by then other groups were already well advanced. In

particular, Dr Katharine High, a pioneer in this field, was preparing to conduct a clinical trial in haemophilia B patients entailing intramuscular administration of AAV vectors encoding human FIX. (Herzog *et al*, 1997; Herzog *et al*, 1999)

Our initial haemophilia gene therapy efforts were focused on a head-to-head comparison of the safety and efficacy of the intramuscular, intravenous and liver (the site of factor IX production) targeted modes of AAV delivery in murine models. I sought help from Dr Andrew Davidoff, a newly recruited faculty member in the Department of Surgery at St Jude, for the challenging task of AAV administration into the portal vein of mice. This was the start of a long collaboration and friendship. In the course of conducting these experiments, Dr Davidoff and I started bouncing ideas off each other, and before he fully realized, he had become integrally involved in the haemophilia gene therapy project.

We discovered that, for the same dose of vector, expression was significantly higher following systemic or portal vein delivery of AAV when compared with intramuscular injections. More concerning was the fact that muscle delivery of AAV was more likely to trigger factor IX antibody production, which in a clinical setting would be disastrous because of its potential to make both gene therapy and FIX protein replacement therapy ineffective. (Nathwani *et al*. 2001) As it happens, intramuscular delivery of AAV in the first trial of this vector in severe haemophilia B patients did not result in any toxicity. However, the plasma FIX levels were not maintained above 1% in any of the seven subjects recruited, despite promising long-term efficacy data in the murine and canine models of haemophilia B. (Kay *et al*. 2000; Manno *et al*. 2003; Herzog *et al*. 1997; Herzog *et al*. 1999; Hagstrom *et al*. 2000)

Murine and canine models of haemophilia B, through efficiently transduced with AAV2 vectors, appeared not to be reliable models for outcomes in humans. We therefore turned to a non-human primate model using rhesus macaques, which like humans are natural hosts for AAV and had been shown to be better predictors of outcomes in humans in relation to oncoretroviral gene transfer of HSC. (Donahue and Dunbar 2001) We demonstrated stable (>5 years) therapeutic expression of human FIX without toxicity following a single administration of AAV2 vector encoding FIX into the portal circulation of NHP. (Nathwani *et al*. 2002; Davidoff *et al*. 2005; Nathwani, Rosales, *et al*. 2011)

At around this time unexpected results were emerging from a second human gene therapy trial, which entailed a bolus infusion of AAV2 vector containing a strong liver specific FIX expression cassette into the hepatic artery of patients with severe haemophilia B. Most worrying was the occurrence of a rise in liver enzymes and elimination of transgene expression in the first subject to be treated at the high dose, ( $2 \times 10^{12}$  vg/kg) whose level of factor IX had risen to 12%. The lower doses evaluated in this study were safe but not efficacious. These events were not observed in animal models even after administration of a 10-fold higher dose than administered in humans. Further immunological studies led to the

hypothesis that the decline in FIX expression and the transaminitis were likely due to memory AAV2 capsid-specific cytotoxic T cells directed against the transduced hepatocytes, which was thought to be a legacy of prior exposure to wild type AAV2. (Manno et al. 2006; Mingozzi et al. 2007) This was very worrying as sero-epidemiological studies showed that >60% of adults had pre-existing immunity to AAV-2 due to prior exposure to wild type AAV2, which could preclude them from future gene therapy trials. (Parks et al. 1970; Blacklow, Hoggan, and Rowe 1968)

We, therefore, turned our attention to AAV8, a new serotype which had been isolated from NHP tissues by the Wilson group. (Gao et al. 2002) Early studies showed that AAV-8 could mediate between 10 to 100 fold higher transduction of murine liver than observed with equivalent numbers of AAV-2 particles. (Gao et al. 2002); (Sarkar et al. 2004); (Grimm et al. 2003); (Davidoff, Gray, et al. 2004), which raised the possibility of therapeutic gene transfer using lower potentially safer doses, thus reducing the risk of transaminitis, whilst easing the burden on vector production. Critically <25% of humans had antibodies to AAV8 and, in addition, it seemed that AAV8 could mediate efficient gene transfer in animals with neutralizing anti-AAV-2 antibodies. (Davidoff, Gray, et al. 2004; Gao et al. 2002) But a major limitation to the clinical use of AAV 8 vectors was the lack of an efficient method for generating clinical grade vector particles.

Returning to the UK in 2000, I was fortunate to have my gene therapy programme generously supported by the Katharine Dormandy Trust, originally set up by Katharine in the 1960s. She had, with extraordinary prescience, included in its objectives research for curative treatment for haemophilia. I was fortunate to recruit Dr Jenny McIntosh to my new research group who diligently developed a scalable method for purification of AAV-8 vectors. (Davidoff, Ng, et al. 2004) This method was later used to generate clinical grade vector in the GMP manufacturing facility at St Jude Children's Research Hospital for use in our pivotal trial. (Allay et al. 2011)

To improve liver transduction beyond that achieved with AAV8 vectors, we capitalised on the observations by Drs Russell and Samulski, who had independently shown that AAV expression cassettes that were half the size of the wild type AAV genome were naturally packaged as two complementary strands within a single AAV particle. This, enhanced the efficiency with which transcriptionally active double stranded proviral DNA were formed resulting in an increase in the transduction efficiency of hepatocytes, muscle and retina by 10-100 fold. (McCarty et al. 2003; Wang et al. 2003; Hirata and Russell 2000; Yang et al. 2002) However, their smaller packaging capacity (~2.5 kb) had limited the clinical application of these self-complementary vectors (scAAV). A new recruit to St Jude at that time, Dr John Gray, helped us to create a more compact (2.1kb) human FIX expression cassette (scAAV-LP1-hFIXco) that met the packaging requirements of scAAV, while maintaining liver-restricted expression through the creation of a new small synthetic liver specific promoter. Another key aspect of this cassette was the alteration of the coding sequence of human FIX by using a subset of codons most

frequently found in highly expressed eukaryotic genes (“codon optimisation”).(Nathwani et al. 2006) The scAAV-LP1-hFIXco vector when pseudotyped with serotype 5 or 8 capsid improved transduction of the murine liver by 20 fold and mediated therapeutic levels of human FIX in NHP following liver targeted administration of significantly lower doses of vector than required with single stranded AAV.

The next important advance in developing our gene therapy strategy for the clinic was the demonstration that the remarkable tropism of AAV 8 for the liver could enable safe and highly effective transduction of the liver following a simple bolus infusion of AAV8 vectors into the peripheral venous circulation of NHP. This validated similar observation in murine models by our group and others. (Nathwani et al. 2007); (Nathwani et al. 2001; Davidoff et al. 2005; Thomas et al. 2004) The peripheral vein route of vector delivery appeared better suited for patients with a bleeding diathesis such as haemophilia B as it was simple and dispensed with the need for invasive procedures such as selective catheterization of the hepatic artery.

Having developed a body of safety and efficacy data for our distinct approach for haemophilia B we embarked on a path to gain regulatory approval both in the UK and USA. Our encounters with the regulators were helpful and instructive. We encounter some resistance from Physicians during ethical review by the Gene therapy advisory committee (GTAC) in the UK. These Physicians had experienced first-hand the catastrophe of contaminated blood products in the haemophilia community. They argued against further experimental therapies for haemophilia patients on the grounds that the current standard of care with recombinant factor concentrate was safe and highly effective resulting in an overall increase in life expectancy to near normal levels. (Darby et al. 2007) In contrast, our gene therapy approach for haemophilia B included several features that had not been tested in humans before, including AAV serotype 8, self-complementary genomic format and systemic administration of vector, and carried the potential risk of integration oncogenesis and cell mediated liver injury amongst others. Patients, in contrast, argued that there was a need to develop alternative treatment options that reduced the need for life-long intravenous injections of factor concentrates.

Despite the reservation amongst Physicians, with support from Ted Tuddenham who was by then the Director of the Katharine Dormandy Haemophilia Centre at the Royal Free Hospital we were able to recruit the first six patients to our trial in the UK. They were divided into 3 cohorts of 2 participants each and received scAAV8-LP1-hFIXco vector by peripheral vein at vector doses of  $2 \times 10^{11}$ vg/kg,  $6 \times 10^{11}$ vg/kg, and  $2 \times 10^{12}$ vg/kg respectively. The low dose subjects who consented, did so in the full knowledge that the dose they would receive was not likely to give them any benefit based on our dose finding studies in animals.(Nathwani, Rosales, et al. 2011) Furthermore, they realized that they would not be able to have a further dose of the same vector as their immune system would then prevent subsequent successful gene transfer with AAV8. Nevertheless, two of our patients, who were motivated purely by

altruistic desire to help the progress of treatment for their condition, volunteered for this dose level. They are, therefore, the real heroes of this story.

To our great surprise we observed stable expression of FIX between 1-2% in both the low dose subjects who, as with all patients in the study, had “severe” haemophilia B, with well-documented baseline FIX activity at <1% of normal. Of the first six patients, four were able to stop prophylaxis completely within 6 weeks after gene transfer. The highest level of transgene expression of between 8-12% of normal was observed in the two subjects treated at the high dose level and remained stable up to six weeks after gene transfer. This level of expression was enough to convert their bleeding phenotype from severe to mild. Patients with mild haemophilia typically have plasma FIX levels of between 5-40% of normal and have very few or no spontaneous bleeding episodes though they are still at risk of excessive haemorrhage after trauma or surgery. Unexpectedly, at around 7 weeks after gene transfer, the first of the high dose patients had a 10 fold increase in liver enzymes associated with a drop in human FIX expression to 2% of normal, which was thought to be due to a cellular immune response to the transduced hepatocytes. We commenced him on prednisolone and this was followed by resolution of the transaminitis but without complete loss of FIX expression. The other high dose patient also developed a slight elevation in of liver enzymes levels over his baseline at around 9 weeks after gene transfer. He too was commenced on prednisolone. His liver enzyme levels promptly returned to baseline values and FIX expression is maintained at 4% of normal levels. He has not required any treatment with FIX concentrates despite living a very active life. He was on thrice weekly FIX prophylaxis prior to gene transfer. (Nathwani, Tuddenham, et al. 2011) In total 10 patients were recruited to this study and in all transgenic FIX activity levels have remained stable over a period of follow-up that extends to 10 years associated with a significant reduction in the annual FIX concentrate usage and frequency of spontaneous bleeding.(Nathwani et al. 2018) Importantly, the quality of life of these individuals has improved dramatically as they are now able to undertake activities that previously provoked bleeds without suffering from bleeding episodes. No late toxicity was observed and neutralising antibodies to FIX were not detected in any patient. On-going monitoring of the liver does not show any evidence of long-lasting damage.(Nathwani et al. 2018)

The gene therapy studies that followed our trial differed in their selection of AAV capsid, configuration of the vector genome, design of the expression cassette, and method of vectors manufacture but the results were consistent with our observations with FIX expression levels in the 5-10% range. Further advances were driven by a new generation of haemophilia B trials using a FIX cDNA containing a naturally occurring gain-of-function mutation in humans characterised by leucine (R338L) instead of arginine at position 338 in the catalytic domain. This mutation enhances FIX activity (FIX:C) by 5 to 8-fold for a given amount of FIX antigen. Therefore, a small increase in plasma FIX antigen levels would lead to a substantial increase in plasma FIX clotting activity. Three clinical trials (BAX 335; SPK-9001; AMT-061) separately

evaluated FIX-Padua in the context of gene therapy and achieved FIX activity levels between 20-40% of normal resulting in zero bleed rates following AAV gene transfer. (Vandamme, Adjali, and Mingozzi 2017) (George et al. 2017) (Von Drygalski et al. 2019) Recently, our group has used an engineered AAV capsid (AAVS3) to mediate higher levels of gene transfer to the liver leading to FIX activity levels in the normal range after a single administration of vector encoding the FIX cDNA containing the Padua mutation, thus raising the prospects of a functional cure.

Encouraged by our success with gene therapy for haemophilia B, we began to look at haemophilia A – a more common bleeding diathesis but technically more challenging because the factor VIII protein is inefficiently synthesized in humans. In addition, the factor VIII cDNA at 7kb is too large (7kb) to be packaged into AAV, which have a maximum packaging capacity of 5kb. Nevertheless, we solved both these obstacles to develop an AAV based gene transfer approach. Expression of human FVIII was improved 10-fold by codon optimisation of the wild type cDNA of human FVIII.(Nathwani et al. 2006; Ward et al. 2011; Radcliffe et al. 2008; McIntosh et al. 2013) 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.

Both these constructs were licensed to Biomarin who have made highly impressive progress with the B-domain deleted FVIII AAV expression cassette, taking it from start of Phase I to full enrolment of a Phase III (>130 severe haemophilia A patients) trial and submission for market authorisation approval within a period of 5 years. The published Phase I/II data from the BioMarin study in shows mean Factor VIII levels of 64% at one year in six patients who received a single intravenous AAV dose of  $6 \times 10^{13}$  vg/kg. Six additional patients treated at a lower dose level of  $4 \times 10^{13}$  vg/kg had mean FVIII activity of around 21% at 1 year after gene therapy.(Rangarajan et al. 2017) FVIII expression in these patients has declined over a 3 year follow-up period by approximately 50% but is still maintained at 4 years at levels that reduce annualised bleeding by >90% despite cessation of FVIII infusion across these two dose levels over time.(Pasi et al. 2020) The BioMarin haemophilia A gene therapy study has been followed by several other clinical trial activity. Most of these studies use of a codon optimised BDD deleted FVIII construct but each trial has used a different serotype and dose range. These differences provide an opportunity for patients with pre-existing antibodies to one serotype to be treated by alternative serotypes.

In summary significant advances have been made in haemostasis over the last 60 years, spearheaded by several impactful discoveries by British Scientists. This has led to dramatic improvements in the treatment for patients with the Haemophilia, that is unparalleled when compared to the other monogenetic disorders. Progress towards gene therapy of the haemophilias at times has been agonizingly slow in the field of gene therapy. However, the

fact that our initial cohort of haemophilia B patients are still benefiting from the one-off gene transfer treatment delivered in 2010 represents an important and encouraging advance in this field. As well as clinical improvement, the study has also generated economic benefits, with a saving of £300K in just the first year of the trial from reduction in FIX concentrate usage, now cumulatively into several millions just from factor concentrates. Our gene therapy success was achieved by an international wide-reaching collaborative effort that brought together many of the key players in the field of gene therapy who were all fully committed to the success of our strategy. Further improvements in this technology have happened at a great pace stimulated by massive investment into gene therapy since our first success reported in 2011 and especially after its follow up report in 2014. There are now 20 trials of gene therapy for haemophilia based on the AAV approach, with more being started regularly.





Figure 1. ET with Professor Macfarlane in 1984 on the occasion when ET was given the Haemophilia Society Macfarlane Award and medal.

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