Gene therapy – are we ready now?

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Abstract
Introduction: Haemophilia therapy has evolved from rudimentary transfusion-based approaches to an unprecedented level of innovation with glimmers of functional cure brought by gene therapy. After decades of misfires, gene therapy has normalized factor (F)VIII and factor (F)IX levels in some individuals in the long term. Several clinical programmes testing adeno-associated viral (AAV) vector gene therapy are approaching completion with imminent regulatory approvals.

Discussion: Phase 3 studies along with multiyear follow-up in earlier phase investigations raised questions about efficacy as well as short- and long-term safety, prompting a reappraisal of AAV vector gene therapy. Liver toxicities, albeit mostly low-grade, occur in the first year in at least some individuals in all haemophilia A and B trials and are poorly understood. Extreme variability and unpredictability of outcome, as well as a slow decline in factor expression (seemingly unique to FVIII gene therapy), are vexing because immune responses to AAV vectors preclude repeat dosing, which could increase suboptimal or restore declining expression, while overexpression may result in phenotoxicity. The long-term safety will need lifelong monitoring because AAV vectors, contrary to conventional wisdom, integrate into chromosomes at the rate that calls for vigilance.

Conclusions: AAV transduction and transgene expression engage the host immune system, cellular DNA processing, transcription and translation machineries in ways that have been only cursorily studied in the clinic. Delineating those mechanisms will be key to finding mitigants and solutions to the remaining problems, and including individuals who cannot avail of gene therapy at this time.

KEYWORDS
AAV, cure, gene therapy, genotoxicity, haemophilia, liver toxicity

1 | HAEMOPHILIA THERAPY: FROM EVOLUTION TO REVOLUTION

Haemophilia A and B are caused by deficiencies of coagulation factor VIII (FVIII) and factor IX (FIX), respectively. Haemophilia therapy has gone a long way since its humble beginnings. Most of that journey led to better ways of replacing the missing factor through incremental improvements in transfusion medicine, biochemistry and molecular biology. In 1840, Samuel Lane made the earliest successful attempt at treating symptoms of hemophilia. The treatment was whole blood and it continued to be the only remotely effective therapy until the 1920s when plasma transfusion was first used to control bleeding, but it did

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not become a common practice until the 1950s. The efficacy of plasma transfusions was still limited because bleeding control required high volumes, running the risk of circulatory overload. Two major technological breakthroughs secured a foothold for more meaningful progress. The first one was the successful isolation of albumin by cold ethanol precipitation in the 1940s by Edwin Cohn, providing the proof of concept that one could isolate therapeutic proteins from plasma on a large scale.2 The next major milestone was the discovery that a cold precipitate that forms following the slow thaw of frozen plasma contains high factor FVIII activity.3 This cryoprecipitate could be easily separated from the rest of the plasma and infused from a closed-bag system, which enabled local preparation and significantly reduced infusion volume, permitting administration of higher doses of FVIII without overloading the patients with fluids. Importantly, cryoprecipitation was able to be integrated into the plasma fractionation workflow. This, together with a number of chromatographic protein purification techniques that came along, allowed for efficient isolation of multiple plasma proteins in the form of concentrates, including FVIII and FIX, which could be freeze dried, stored in a refrigerator and used at home. The two seminormal processes, cold ethanol fractionation and cryoprecipitation are still used in modern plasma fractionation.4

Refinements in the chromatographic purification techniques produced an array of FVIII and FIX clotting factor concentrates, whose purity increased over time from < 1% of total protein to ~99% purity by the end of 1980s.5 Inadvertently, large-scale manufacture from plasma pools, including plasma from as many as over a hundred thousand individuals and the widespread use of clotting factor concentrates, facilitated the spread of hepatitis B and C, and human immunodeficiency viruses (HBV, HCV and HIV), which altogether took lives of many thousands of individuals in the haemophilia community.6 The catastrophe stymied the adoption of prophylaxis and conditioned the patients for many years to feel that danger was lying in wait.

The generation of high-purity FVIII and FIX concentrates was pivotal for cloning the F8 and F9 genes in 1984 and 1982, respectively.7–12 Efforts were well afoot to that end and intensified when the HIV disaster unfolded, which substantiated the urgent need to develop safer treatment products, leading to regulatory availability of the first recombinant FVIII and FIX products in 1992 and 1997, respectively. Also, the successful cloning and expression of the F8 and F9 genes raised the prospect of ultimately designing a functional cure for haemophilia in the form of gene therapy.

In the 1990s and 2000s, growing access to safe recombinant and plasma-derived clotting factor concentrates, which had largely rebuilt trust with the community thanks to the unblemished viral safety record since the introduction of viral reduction measures in the manufacture, prompted a reappraisal of prophylaxis in recognition of the unmet need for better bleeding protection.13 Prophylaxis has improved outcomes and become a standard of care in children, but did so at the cost of increased treatment burden and fell short of eliminating all joint damage. Peaks and troughs in factor levels mean that patients still spend a considerable amount of time at factor levels that are incapable of preventing all spontaneous or subclinical bleeds. The 2010s ushered into the market a wave of extended half-life (EHL) clotting factor concentrates that have facilitated prophylaxis by affording fewer infusions and higher troughs, especially for patients with haemophilia B.14 The same decade also saw a number of non-factor therapies enter the clinic, one of which, the bispecific antibody emicizumab, has already been commercialized and improved outcomes in patients with and without inhibitors, which are currently the most serious complication of treatment.15 All or most of these new treatments offer easier (subcutaneous) and less frequent administration, and for the first time break the paradigm of seesaw pharmacokinetics, representing a molecular revolution in tapping into the coagulation system to restore hemostasis.16 However, they have vastly different mechanisms of action, do not normalize hemostasis and cannot be measured using the legacy assays that have long been established in the factor replacement therapy.17 Thus, the pursuit of better modalities and, ultimately, a cure have persisted.

2 HOPES AND LETDOWNS IN GENE THERAPY FOR HEMOPHILIA

The last decade also saw the first evidence that gene therapy for haemophilia A and B has the potential to normalize factor levels in the long term due to several adeno-associated viral (AAV) vector gene therapy programs making some headway. The path toward that point has been long, winding and littered with roadblocks. The concept of gene therapy emerged 50 years ago.18 The idea of using a virus as a gene transfer tool was born soon after when Terheggen and coworkers made an attempt to correct arginase deficiency by intravenous administration of the Shope papillomavirus into three hyperargininemic patients.19,20 They failed, but the idea remained attractive and several years after the F8 and F9 had been cloned, the nascent field of haemophilia gene therapy was using retroviral vectors for successful gene transfer in primary human cell lines that were then implanted into small and large laboratory animals.21–24 Some of those early experiments produced long-term but low-level expression in the animals, or vice versa. Yet, the results were reassuring enough to continue testing several viral vectors throughout most of the 1990s. Systemically administered adenoviral vectors produced transient therapeutic and supratherapeutic levels of factors for weeks and months in mice and dogs, but immune responses were limiting the duration of expression and precluded repeat administrations.25–27

Advance to the clinic became tangible when the prototypical AAV2 vector induced stable FIX expression upon intramuscular or intraportal injection in mice, and subsequently in dogs with haemophilia B.28–33 Although AAV looked like a favourite to enter clinical trials, the first human gene therapy study evaluated a non-viral approach by implanting autologous fibroblasts transfected with the FVIII gene into omentum, closely followed by a trial that systemically delivered a retroviral vector encoding FVIII.34–36 Both misfired, producing transient factor level increases within the moderate haemophilia range in some participants.37,38 Two AAV2 haemophilia B trials followed, using the vectors that showed promise in mice and dogs, one injecting the vector in the muscle, the other in the portal vein.39,40 In the muscle
After decades of disappointments, the first gene therapies for haemophilia A and B are nearing commercialization, and thus the long-anticipated cure has come within reach but not for everyone. Data from larger groups of patients enrolled in Phase 3 studies have substantiated several concerns about variability, the durability of efficacy, and safety that emerged at earlier stages. In all trials, FVIII and FIX expression is highly variable, to the point that means and medians poorly represent levels achieved by individuals, from no response at all to moderate increase to levels across mild haemophilia and normal ranges, and far above the upper limit of normal. Most individuals show elevations of liver enzymes, particularly alanine aminotransferase (ALT). These elevations are the most common, albeit asymptomatic, adverse event, typically managed with corticosteroids (as in the original 2010 trial) and other forms of immunosuppression. They are generally considered mild and transient, 1-3-fold above the upper limit of normal, but in some participants went up as high as 10–20 times over the upper limit of normal or persisted for months and as long as over a year. In haemophilia B trials, some but not all of these cases correlate with cytotoxic T cell response toward capsid proteins and they variably respond to immunosuppression. Haemophilia A studies rarely found evidence of immunototoxicity and responses to immunosuppression are even less consistent. Yet, in haemophilia A individuals, ALT seems to rise more often and be somewhat dose-dependent. In a Phase 3 study of valoctocogene roxaparvovec (BMN 270) administering the largest vector dose in the field (6e13 vg/kg), 115 out of 134 dosed individuals had ALT elevations, 106 of whom received corticosteroids, and 39 other immunosuppression. The durability of efficacy has been clearly different between FVIII and FIX studies. For FIX, transgene expression has been durable. Participants of the original 2010 trial have maintained stable FIX expression and more recent trials using FIX-Padua AAV constructs seem to be following the same pattern. In contrast, FVIII levels continue to decline and after 5 years have dropped five-fold from the mean 57.7 (median 47.8) to 11.6 (8.2) IU/dL (measured with chromogenic substrate assay) in the longest-running FVIII trial. These elevations are the most common, albeit asymptomatic, adverse event.

In 2010, after several years of hiatus, gene therapy for haemophilia returned to the clinic with a verve, when several haemophilia B patients received AAV8-FIX, and whose FIX expression was rescued with a course of corticosteroids when their transaminases started to rise. This pioneering effort has informed a slew of subsequent trials evaluating AAV vectors encoding FIX or FVIII. They all administer AAV vectors targeting the liver in a single bolus intravenous injection. The initial haemophilia B studies delivered transgenes encoding wild-type FIX and saw FIX increases within moderate and lower mild ranges. Better results came with the inclusion of the hyperactive Padua variant FIX, which harbours a single amino acid substitution and has since been used in all leading haemophilia B trials. The change revs up FIX activity 5-10-fold, affording higher FIX levels without increasing the vector dose. Thus, the same vector doses as those used in wild-type FIX trials boosted the achieved FIX increases to upper mild and normal FIX ranges. The same team that ushered haemophilia B gene therapy back into the clinic designed several AAV vectors encoding B domain-deleted (BDD) FVIII, one of which was selected for clinical development and became BMN 270 in 2014, now in Phase 3. It was the first clinical trial that achieved normal FVIII levels in some individuals, and several other studies soon followed having similar success with different vectors. Four clinical programs have advanced to Phase 3 with some early phase studies having multiyear data.
persist for at least 15 years, precludes repeat dosing in patients with suboptimal response, even with other serotypes.74,75 Potential solutions, such as plasma-exchange or proteolytic antibody depletion are at early stages of development or difficult to integrate in routine practice. Conversely, patients on the other side of the variability spectrum are faced with the risk of phenotoxicity insofar that supraphysiological factor levels may lead to thrombosis. High factor levels prompted the recent pause of a FVIII gene therapy program and prophylactic anticoagulation in some individuals as well as acute anticoagulation in a participant of a FIX gene therapy trial.76–78 This issue also begets discussion of the ‘right’ target factor level (or range) for gene therapy. One train of thought maintains that gene therapy should aspire to cure and the cure is factor level in the normal range (50–150%). The teleological justification would be that evolution favoured the FVIII 50–150% range because we need it to prevent any bleeding whatsoever. However, teleological arguments are not necessarily correct and supporters of targeting subnormal levels lament the loss of cardioprotective effect with higher expression and point to the elimination of joint bleeds seen at levels over ~15%.79–81 Bleeding rates observed in the gene therapy studies to date have supported the latter view.

Since the seminal discovery of cytotoxic T cell-mediated killing of transduced hepatocytes, nonclinical research on immune responses to AAV gene therapy has painted a much more complex picture, elements of which have already manifested in the clinic pointing to potential mitigants.41,44,82 Toll-like receptor 9 (TLR9)-mediated sensing of vector DNA cargo has emerged as a key mechanism triggering immunotoxicity and expression loss.83–85 TLR9 is an innate immune sensor of unmethylated CpG sequences in DNA, which are a pathogen-associated molecular pattern (PAMP). The most advanced clinical programs stripped their vectors of those elements, but the latest three failed trials inadvertently enriched their DNA constructs in CpG sequences leading to FIX expression loss within three months from dosing that could not be rescued with immunosuppression. Removal of CpG sequences from non-coding elements of the expression cassette, such as promoters and inverted-terminal repeats (ITRs), is difficult. The remainder potentially contributes to residual immunogenicity of otherwise CpG-depleted constructs, manifesting as transaminase elevations that variably respond to immunosuppression and correlate with vector dose.84,86–88 Additional causes of these elevations, such as vector impurities (e.g. empty or defective capsids, encapsidated extraneous DNA, adventitious agents) have not been ruled out.89–91 AAV transduction is an extremely inefficient process, with ~99% of vector perishing in the cellular garbage disposal pathways before settling in the nucleus.72,92,93 Excess empty capsids could further burden the already strained mechanism.90 The discontinued study of BAX 335 suggested that interleukin-6 receptor (IL-6R) is a promising target for AAV immunotoxicity mitigation.83,84 Non-clinical research has identified several other targets that may be fertile for clinical translation, including interleukin-1 (IL-1), interleukin-6 (IL-6), type I interferon, CD40-CD40L co-stimulation and the mammalian target of rapamycin (mTOR).82,94,95

In FVIII trials, presumably nonimmune transaminase elevations and the relatively slow (multiyear) decline of FVIII levels may originate from unique challenges of the FVIII biosynthesis and FVIII AAV vectorology. Unlike FIX, FVIII is primarily produced by the liver sinusoidal endothelial cells (LSECs), not by hepatocytes.96 Yet, AAV vectors encoding FVIII target hepatocytes, and thus FVIII transgene expression is ectopic. FVIII is a large and complex glycoprotein, notoriously difficult to express in heterologous cells even compared to other similarly sized and structured proteins, the reasons of which are incompletely understood. Several bottlenecks have been proposed to explain the poor FVIII expression, including transcription-repressing elements in the F8 gene, tendency to misfold, aggregate and degrade, the requirement for specific receptor complex (lectin, mannose binding 1/multiple coagulation factor deficiency protein 2, or LMAN1-MCFD2) to traverse the cellular secretory pathway and poor stability in the absence of von Willebrand factor (VWF).97,98 Misfolded FVIII activates unfolded protein response, which can lead to cellular stress and death.99,100 FVIII tolerates engineering within the B domain (which is naturally excised during activation anyway), removal of which has improved expression and permitted inclusion in AAV cassettes, producing constructs of ~5 kb as compared to >7 kb for the full-length FVIII.101 However, BDD-FVIII transgenes still exceed the optimal AAV capsid packaging limit. Vector packaging efficiency dramatically drops with transgene extension beyond 4.7 kb, which results in a mixture that consists mostly of vectors harbouring truncated cassettes.102,103 Some fragmentated AAV undergo repair in the nucleus and may be assembled into functional monomeric and concatemeric circular episomes by the cellular DNA processing machinery, which is essential for the establishment of persistent transgene expression.104–108 However, this form of gene transfer results in extra loss of transgene from the administered vector dose and adds to the already tall order of managing a sudden influx of hundreds of thousands of vector particles per hepatocyte, the impact of which is unknown. Thus, FVIII gene therapy is a compromise between transgene cassette size and vector dose necessary to induce meaningful transgene expression.

4 | REDUCING UNCERTAINTY

The causes and mitigants of wide variability, waning efficacy (for haemophilia A) and liver toxicity have not been sufficiently studied, and thus the advance of gene therapies toward commercialization has outpaced our fundamental biological understanding of AAV gene transfer in humans (Table 1). This will need to be rectified to ensure safety and the best possible outcomes at the current state of the art. Minimizing AAV ‘wastage’ throughout its journey to the nucleus and improving factor expression could lower vector doses by logs, thus potentially further reducing toxicities. The dose is likely critical as toxicities grow in frequency and severity with increased vector dosing, and in gene therapy for other conditions that delivered vector doses exceeding 14 vg/kg (higher than in any haemophilia study) included complement activation, cytopaenias and severe hepatotoxicity, likely representing part of the continuum of clinical immune responses. More transparency and data sharing as well as timely publication of animal and clinical data, including negative results, would help find solutions.
Failed studies may offer important learnings as exemplified by the recent publication of the discontinued BAX 335 program.84 Liver biopsies at different time points after vector dosing will be critical to understanding AAV gene transfer biology in humans. Also, the field needs standardization. For instance, clinical trials use two types of assays to measure pre-existing immunity to AAV vectors: ELISA-based total antibody assay and transduction inhibition assay, neither of which has been standardized. This is more obvious and troubling for the transduction inhibition assay because the results determine anti-AAV neutralizing antibody titres and eligibility for gene therapy. Clinical trials have used vastly different assay conditions (if reported at all), making the results incomparable across the field. For example, different multiplicities of infection (MOI) that have been used by one haemophilia A and one haemophilia B trial could produce over ~60-fold different titres for the same vector (assuming all other conditions were the same). Vector production and purification may add to the variation because inactive viral particles may lead to underestimated antibody titres. Nevertheless, vector dose and serum volume are some of the key parameters of the transduction inhibition assay and changes in reporting these would bring some clarity. One way of minimizing the variation could be to report the presence of transduction inhibitors as the number of AAV particles that are neutralized per volume of serum instead of neutralizing antibody titres.109 Another assay issue concerns discrepancies between one-stage (OSA) and chromogenic substrate assays (CSA) when measuring transgene-expressed factor levels. OSA overestimates coagulation in FVIII gene therapy by ~1.6-fold because transgene-expressed BDD-FVIII speeds up early activation of factor X but does not increase overall thrombin generation, so CSA seems more reliable.110,111 Discrepancies have also emerged in FIX trials, but these appear to be inherent to FIX-Padua enhanced kinetics and unrelated to in vivo transgene expression.112–114

The long-term safety of AAV gene therapy for haemophilia has so far been favourable both in the clinic and large animal models but needs continued attention.115–118 AAV have long been misperceived as non-integrating vectors due to the formation of episomes and the low rate of chromosomal integration estimated between 1 and 1.0%. However, in absolute terms, this may still result in millions of integration events in the liver with vector doses ranging from trillions to quadrillions (e12–e15) of particles, raising questions about genotoxicity and oncogenicity risks.119,120 Although the evidence of risk is limited to neonatal or liver-damaged rodents, it merits vigilance and long-term, preferably lifelong monitoring of gene therapy recipients.120–124 Comprehensive genetic analysis of hepatocellular carcinoma (HCC) recently found in a FIX gene therapy trial participant found no evidence that gene transfer caused the malignancy, but integration events were found in 0.027% of cells in the tumour sample, confirming that integrations do occur.125 Importantly, despite their hepatotropism and liver-specific expression AAV vectors may transduce other tissues, so monitoring should be geared toward detecting other potential toxicities.126,127 A framework of known unknowns has been proposed to organize thinking around the short- and long-term safety, and help address the uncertainties.119 Hopefully, the growing attention and calls to action on these matters will galvanize the field to pursue answers.120,128–130

### TABLE 1 Key challenges facing AAV-mediated gene therapy for haemophilia

| Efficacy | Safety |
|----------|--------|
| • Extreme variability and unpredictability of expression | • Prolonged corticosteroid usage and side effects |
| • Declining FVIII levels | • Nonimmune (?) liver transaminase elevations |
| • Vector redosing and treatment of patients with suboptimal factor expression | • Monitoring genotoxicity |
| • Eligibility of individuals with preexisting anti-AAV antibodies | • Phenotoxicity (extremely high factor expression) |

**Related research questions requiring further study**

- What causes the apparently nonimmune liver transaminase elevations?
- What is the role of non-steroid immunosuppression in managing immunotoxicity?
- Is FVIII expression sustainable in the long term?
- Do hepatocytes struggle with FVIII biosynthesis (which normally takes place in liver sinusoidal endothelial cells)?
- Does transgenic protein or vector overload contribute to toxicities?
- Can AAV be retargeted to the endothelium?
- Can transduction efficiency be improved to use lower vector doses? Is it druggable?
- Can expression variability be reduced?
- Does factor level normalization abolish the cardioprotective effect of haemophilia?
- Can AAV vector integration trigger oncogenesis in humans? Can those events be detected?
- Can toxicities be further reduced by improved vectorology? Does vector quality (the presence of empty or defective capsids, adventitious agents) contribute to toxicities?

Gene therapy has long held the promise of the ultimate cure that would provide lifelong haemophilia correction upon a single intervention. Does the state of the art match the dream? It might for some because haemophilia B individuals continue to have stable factor levels for years. For haemophilia A, the ‘cure’ appears temporary. Despite imperfections, first market approvals are imminent. Several subgroups in the community are missing out on the opportunity altogether, including females, children, patients with current or past inhibitors, and, most of all, individuals with preexisting anti-AAV immunity. Globally, 85% of
people with haemophilia may not have ready access to gene therapy based on the current price estimations, while it would offer the only viable solution for those living in resource-limited jurisdictions without immediate prospects for improvement.\textsuperscript{131–135} Future nonclinical, clinical and organizational efforts should aspire to include those long underserved and underprivileged groups. As a new technology, gene therapy presents unique challenges for healthcare providers who will be introducing and delivering it to the patients. Initiatives toward formulating principles and providing guidance are afoot.\textsuperscript{136–139} AAV gene therapy has proven itself as a powerful platform for therapeutic gene transfer, as evidenced by almost 150 clinical trials registered to date and several regulatory approvals, but it has room for improvement and will continue to evolve.\textsuperscript{140–142} Due diligence in addressing uncertainties will be paramount for the haemophilia community, who have endured a difficult safety legacy and failed hopes.

Ideal gene therapy for haemophilia will be the endpoint of the perennial struggle to close the gap between how much haemostatic correction a therapy can provide and how much is needed to live a life independent from treatment. The current state of the art brings us remarkably close to that goal, but actually getting there will take more time and innovation.

CONFLICTS OF INTEREST
RK conceived of and wrote the manuscript. RK received research funding from Bayer Hemophilia Awards Program.

DATA AVAILABILITY STATEMENT
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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