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SUPPLEMENT ARTICLE

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Bioengineered molecules for the management of haemophilia: Promise and remaining challenges

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Abstract

Recombinant DNA technology has led to accelerating introduction of novel therapeutics for the treatment of haemophilia. This technology has driven the development of recombinant clotting factors, extended half-life clotting factors, alternative biologics to promote haemostasis and enabled the launch of the gene therapy era for haemophilia. At the core of this technology is the ability to study the structure and function of the native molecules and to apply rational bioengineering to overcome limitations to the existing therapies. Through the study of haemophilia-causing mutations, site-directed mutagenesis, detailed structural models and a wide repertoire of animal models, new bioengineering strategies are helping overcome some of the remaining limitations and challenges of traditional clotting factor concentrates. Some of these bioengineering strategies are now being partnered with improvements in vectorology leading to the first wave of successful gene therapy approaches. This study will review past and present bioengineered molecules that are advancing care for haemophilia as well as novel approaches that promise to continue to improve care and outcomes for patients with haemophilia.

KEYWORDS

bioengineered, factor IX, factor VIII, gene therapy, haemophilia, recombinant

1 | INTRODUCTION

The initial wave of recombinant clotting factors provided a recombinant facsimile of their plasma-derived counterparts with remarkably similar biochemical, pharmacokinetic and pharmacodynamics properties.[1] Recombinant clotting factors have consistent manufacturing and processing liberated from the uncertainties of securing source plasma with at least the potential for an unlimited supply. With increased supply, there has been an increase in the availability of clotting factors and increased utilization of prophylaxis in developed and developing countries.[2,3] In many health systems, this has driven down the unit costs of replacement therapy and allowed for more aggressive prophylaxis regimens targeting higher trough levels and lower annualized bleed rates.[4] Extensive global safety reviews over the past 25 years have demonstrated no infectious pathogen transmission, no safety signals from adverse event reporting and no evidence of increased rate of inhibitors in previously treated patients (PTPs).[5,6]

The remaining challenges in the recombinant era include barriers to the adoption and adherence to prophylaxis. This can be attributed to the burden of a requirement for consistent venous access, time and costs of primary prophylaxis initiated in infants and young children and continued throughout adulthood.[7] In addition, recombinant factor VIII (rFVIII) products have not been shown to reduce the risk of inhibitor development among previously untreated patients (PUPs).[8] This "natural" immune response to exposure to a foreign protein has been observed for all plasma-derived (pdFVIII) and rFVIII products with some studies demonstrating a significantly increased rate for the rFVIII.[9] There is considerable variability among patients with respect to phenotypic and pharmacokinetic variability. Even among those who have benefitted from early introduction of continuous prophylaxis, annualized bleed rates are not zero for all patients with joint disease still appearing in young adults followed over 25-30 years.[10] Further improvements in outcome can be achieved if patients have better bleed control. This can occur through improved adherence, which can be facilitated through strategies that reduce

the burden of administration—reduced dosing frequency, increased availability at reduced cost and application of alternative modes of delivery (eg, subcutaneous routes). The challenge of inhibitors can be overcome with molecules with reduced or even absent immunogenicity or through highly efficacious bypassing activity.[11]

TABLE 1 Bioengineering strategies for enhanced biologics

cDNA modifications
B domain deletion/truncation
Amino acid substitutions
Interactive site modification
Novel conjugation sites (eg, targeted introduction of cysteine)
Targeting immunogenic epitopes
Single-chain molecules
Altered post-translational modifications (PTM)
Cell line choice (mammalian vs human)
Novel glycosylations
Production efficiency/consistency
Reduced aggregates, optimized PTM
Polymer conjugations
Polyethylene glycol
XTEN
Fusion proteins
Fc fragment
Albumin

Basic research has provided insights on structure and function characterizations throughout the life cycles of FVIII and FIX including biosynthesis, macromolecular interactions, activation/inactivation, and clearance. Through the study of haemophilia-causing mutations, site-directed mutagenesis, detailed structural models and a wide repertoire of animal models, new bioengineering strategies are helping overcome some of the remaining limitations and challenges of traditional clotting factor concentrates.[12] Bioengineering strategies of biologics that can be applied to FVIII and FIX are summarized in Table 1. Partnering these bioengineering strategies with improved viral vectors has produced the first wave of successful gene therapy approaches.[13] This study will review past and present bioengineered molecules that are advancing care for haemophilia as well as novel approaches that promise to continue to improve care and outcomes for patients with haemophilia.

2 | BIOENGINEERING OF FVIII

2.1 | B domain-deleted FVIII—the first bioengineered FVIII

Expression of rFVIII and its stability in plasma is limited by several mechanisms as summarized in Table 2. One of the first innovations was removal of the FVIII B domain, accounting for ~38% of the primary cDNA sequence, without impairing important FVIII functions such as its affinity for von Willebrand factor (VWF) or its procoagulant activity.[14,15] This improved the yield of rFVIII in cell culture

TABLE 2 Bioengineering factor VIII for improved expression

Impediment to FVIII expression	Bioengineering strategy	Outcome	Reference
Inefficient mRNA expression	Deletion of B domain	17-fold ↑ mRNA. 30% ↑ secretion	Toole et al[15] Pittman et al[14]
ER retention/BiP binding	F309S (A1) mutation	Two- to threefold ↑ secretion	Swaroop et al[51]
ER-Golgi facilitated transport	226aa B domain/6 N-glycans (226/N6)	Five- to 10-fold ↑ secretion	Miao et al[36]
? Oxidative stress	ΔC1899-C1903 disulphide loop	Twofold ↑ secretion	Selvaraj et al[28]
Transcriptional silencers/inhibitory motifs in F8 cDNA	Codon optimization	29- to 44-fold ↑ in expression	Ward et al[24]
F8 size constraints for safe AAV-mediated gene therapy	rAAV-HLP-codop-hFVIII-V3	Supraphysiologic expression in HA mice (732 ± 162% of normal)	McIntosh et al[37]
Optimal furin cleavage/faster A2 domain dissociation	Modified furin cleavage site variants—mostly single-chain forms	Twofold ↑ procoagulant activity/two- to fourfold ↑ expression than BDD	Nguyen et al[39]
ER chaperone binding and UPR induction	Human-porcine hybrid FVIII (ET-3) with "high expression" porcine FVIII A1 and ap-A3 seqs.	10-to 100-fold improved biosynthesis resulting from ↑ secretion	Brown et al[40]

expression systems as a result of significantly increased mRNA and increased translation. This was eventually characterized in clinical studies and demonstrated that clinical efficacy and rates of inhibitor formation in PUPs with haemophilia A were similar to that observed with full-length rFVIII products.[16] This molecule has since become the backbone for many additional biochemical modifications to extend its half-life including Fc-fusion and PEGylation, currently in clinical use. However, its properties have been best exploited to advance gene therapy efforts. The smaller size of the B domain-deleted FVIII (BDD-FVIII) cDNA helps facilitate more efficient packaging into viral vectors, such as adeno-associated virus, which otherwise could not accommodate a full-length FVIII cDNA.[13] With similar biochemical characteristics to full-length FVIII, BDD-FVIII has now been incorporated into all active gene therapy programmes for haemophilia A.

2.2 | Codon optimization

The genetic code, a triplet of bases of nucleotides, or codon, encoding for 1 amino acid, is degenerate. As such, with the exception of 2 amino acids, Met and Trp, each amino acid can be encoded by one of several nucleotide triplet combinations, so-called synonymous codons.[17,18] Codon usage bias varies between organisms related in part to organism-specific differences in populations of cognate tRNAs.[18] Thus, frequently used codons will be translated more rapidly than infrequently used codons. Codon optimization involves replacing rare codons with frequently used ones to increase the efficiency of protein expression. This has been used in one currently approved EHL-rFIX (rFIX-FP)[19] and has become a mainstay of FVIII and FIX expression constructs used in gene therapy. Codon optimization applied to BDD-FVIII can substantially increase the expression.[20] The improvement in FVIII expression efficiency can allow lower doses of viral vector and facilitate achieving higher levels of plasma expression. However, a number of studies have demonstrated that synonymous codon changes can have unanticipated effects on protein conformation and stability, altered post-translational modifications and protein function.[21]

Codon usage is thought to determine the elongation rhythm, causing ribosomes to slow down or pause at certain sites, thereby modulating sequential folding events that occur co-translationally.[18,21] This differential synonymous coding is a secondary code guiding in vivo protein folding, and thus, it should not be surprising that studies have identified examples of synonymous codon changes affecting protein activity, interactions with drugs and inhibitors, phosphorylation profiles, sensitivity to limited proteolysis, spectroscopic properties, propensity for aggregation and protein structure (reviewed in Ref. 18). Notably, a naturally occurring synonymous codon change within the factor IX gene, c.459G>A (Val107Val), has been shown to be responsible for a case of haemophilia B[22] by significantly slowing FIX translation and affecting its conformation. Codon optimization can also disrupt alternative start sites for translation, generating new sites that would encode novel peptides that could trigger immune reactions or interfere with normal cellular functions.[21] An alternative to codon optimization is codon harmonization, seeking to identify and maintain regions of slow translation thought to be important for protein folding.[21,23]

Codon optimization strategies are not standardized, and it is unlikely that any 2 codon-optimized FVIII or FIX constructs would be identical in sequence. Given the potential adverse impact of the various synonymous mutations, it is therefore critical to characterize fully the biochemical profile of the expressed protein as well as consider the potential for cellular stress effects through the impact of intracellular misfolded protein. Shestopal et al[20] performed extensive biochemical characterization of a codon-optimized BDD-FVIII expressed in Chinese hamster ovary cell lines. Notably, the codon usage bias of Chinese hamsters is not very different from humans.[17] They observed that the codon-optimized BDD-FVIII was expressed sevenfold higher, similar to prior experience with another codon-optimized BDD-FVIII,[24] without evidence for a significant effect on its structure and function properties. There were some differences observed for post-translational modifications including N- and O-glycosylation at specific sites and degree of Tyr-1680 sulphation. They also noted an average specific activity that was 1.5-fold higher than wild-type FVIII and higher ratios of activities as measured by clotting assay or thrombin generation compared to chromogenic assay. They attributed this to the quality of the preparations due to the higher protein concentrations and a higher abundance of single-chain BDD-FVIII. However, this is an interesting observation given results from a recent haemophilia A gene therapy study. A phase 1/2 study of BMN270, an AAV5 gene transfer of a BDD-FVIII in severe haemophilia A, produced sustained mean and median FVIII levels of 93% and 77% respectively over a 1-year observation period. Subjects demonstrated a consistent correlation of one-stage/chromogenic FVIII activity of 1.65, which would not have been expected based on observations with BDD-rFVIII produced recombinantly in cell lines.[25] Future strategies for codon optimization will be driven by bioinformatics insights that are improving prior databases of codon usage tables[17] and through insights on the potential impacts of rare codon clusters on secondary protein structure.

2.3 | Rational bioengineering through targeted mutagenesis

Expression of FVIII in heterologous mammalian cells is about 2-3 orders of magnitude lower than similarly sized proteins.[26] This inefficiency in expression contributes to low yields of recombinant protein production and must also be overcome in order to achieve efficacious plasma levels with haemophilia A gene therapy. Some of the factors that contribute to this inefficiency are summarized in Table 2 and include inefficient expression of the mRNA coupled with misfolding and degradation of a sizeable portion of the primary translation product[26,27]; retention of FVIII within the endoplasmic reticulum (ER) through interaction with several ER chaperones; formation of multiple disulphide bonds[28]; requirement for a facilitated transport mechanism comprising the mannose-binding lectin LMAN1 and MCFD2 for efficient transport of FVIII from the ER to the Golgi[29,30]; limited proteolysis within the Golgi, wherein a

predominant cleavage occurs at the paired amino acid cleaving enzyme (PACE or furin) recognition motif at the carboxy terminus of the B domain yielding a heterodimer FVIII polypeptide consisting of heavy and light chains associated through a metal ion-dependent association; and the predominant heterodimeric form that is secreted requires stabilization by VWF.[31]

Over the past decade, insights from this analysis of FVIII structure and function have delineated rational bioengineering strategies to overcome many of the limitations of FVIII expression (summarized in Table 2). As these bioengineering efforts have targeted different aspects of FVIII biosynthesis, folding, chaperone interactions, proteolytic processing, secretion and stabilization, combining these targeted modifications can have an additive effect with bioengineered variants exhibiting up to 50-fold improvement over wild-type FVIII expression. Of particular note is a B domain truncated FVIII variant that includes 226 amino acids of native B domain and retains 6 potential consensus sites for N-linked glycosylation (226/N6). This construct exhibited increased mRNA, improved ER-Golgi transport and reduced cellular (ER) stress.[32-36] As a single targeted modification, it exhibited up to 10-fold improved expression in preclinical experiments in vitro and in vivo, including as part of viral vector-mediated gene transfer. The challenge for the 226/N6 construct is that the overall size of the cDNA constrains efficient packaging within AAV vectors. McIntosh and colleagues addressed this limitation by replacing the B domain sequence with a 17 amino acid peptide that contained 6 putative N-linked glycosylation sites.[37] Following codon optimization, this construct could be packaged more efficiently within rAAV vectors with a human liver-specific promoter as rAAV-HLP-codop-hFVIII-V3 (or V3). V3 was observed to be safe and efficacious within mice and non-human primates and is now being tested within human subjects in a phase I/II clinical trial for haemophilia A (NCT03001830, www.clinicaltrials.gov). The investigators will test whether the higher efficacy observed with this construct in preclinical studies will allow satisfactory efficacy in humans at vector dosage levels that have already been observed to be safe in humans as part of AAV8 gene therapy for haemophilia B.

2.4 | Bioengineering through comparative biology

Comparative biology is yielding some new insights for targeted bioengineering. Whereas human BDD-FVIII is secreted predominantly as a heterodimer, canine BDD-FVIII is secreted predominantly as a single-chain polypeptide. Canine BDD-FVIII has also been shown to be more stable and has a higher specific activity compared to the human form and that this could be attributed to suboptimal cleavage of the canine BDD-FVIII by furin.[38] Nguyen and colleagues hypothesized that deletion of part or all of the furin cleavage recognition sequence could increase the proportion of single-chain human BDD-FVIII.[39] Two deletion variants were secreted primarily as single-chain forms and had twofold higher procoagulant activity compared to BDD-FVIII when expressed from cell lines and achieved 2- to fourfold higher expression in mouse plasma after AAV vector delivery. In another example of comparative biology, Brown and colleagues used insights from characterization of porcine BDD-FVIII.[40] They generated a human-porcine hybrid designated ET3, consisting of human FVIII sequences within the FVIII A2, C1 and C2 domains and porcine sequences in the A1 domain and the activation peptide-A3 domain. The overall amino acid sequence substitution represented about 9% of the overall sequence but conferred between 10- and 100-fold improved biosynthesis in cell line expression systems. When packaged into an AAV vector, AAV-ET3 achieved correction of FVIII in haemophilia A mice at lower vector doses than could be achieved with 100% human FVIII sequence constructs.

2.5 | Ancestral comparative biology

The bioengineering approaches described thus far are optimization of a protein through rational design. An alternative approach to protein optimization is through ancestral sequence reconstruction (ASR). This approach is based on the observation that extant FVIII orthologs have molecular, cellular and immune recognition properties that vary between species (eg, murine, canine, porcine and human). The diversity is hypothesized to represent adaptive traits through pressures of natural selection to promote haemostatic balance. The analysis of these differences can lead to insights towards leveraging bioengineered forms with enhanced functional properties. Zakas et al[41] have gone beyond the work based on ortholog scanning that led to the ET3 FVIII construct and have generated higher resolution mapping of FVIII protein sequences through comparisons of sequential phylogeny branches. This led to the identification of ancestral FVIII constructs which demonstrated significantly higher FVIII production compared to human FVIII. They hypothesize based on observations with porcine FVIII and the ET3 FVIII constructs that the conferred increased secretion expression is mediated by reduced engagement of unfolded protein response (UPR) pathways. This ASR approach has also yielded insights on ancestral FVIII constructs with enhanced specific activity, stability and altered immune reactivity. Insights from this analysis could lead to targeted modification of FVIII that may yield superior rFVIII versions or gene therapy constructs.

3 | CHALLENGES TO BE ADDRESSED WITH BIOENGINEERED FVIII CONSTRUCTS

3.1 | One-stage/two-stage assay discrepancy

The clinical implementation of BDD-FVIII has not been without some challenges in clinical assays and potency assignment. BDD-FVIII activity may be up to 50% lower in one-stage aPTT assays compared to chromogenic assays.[25,42] The mechanism for this discrepancy has yet to be fully explained. There may be subtle conformational changes resulting from the B domain deletion that alter phospholipid interactions.[43,44] The potency estimation of BDD-FVIII is sensitive to factor X activation in the chromogenic assay with discrepancies noted between various chromogenic assay kits. There may also be differences in the profile of FVIII activation by thrombin within each

of the 2 types of assays that contribute to the discrepancy between BDD-FVIII and full-length FVIII. This discrepancy can be abrogated by altering the composition of the source phospholipid such that phosphatidylserine content is maintained below 10%. In 2003, due to these assay discrepancies and effects on potency assignment, a commercial BDD-FVIII (Xyntha/ReFacto AF, Pfizer, Boston, USA) was reformulated with 20% more drug product than the original formulation to achieve the stated potency.[45] Calibration of the one-stage assay with a product-specific standard has also been recommended.[25] However, this has not eliminated some patient-specific factors that can contribute to discrepancy when assaying postinfusion plasma samples.[46]

Recombinant FVIII single chain (Afstyla, CSL Behring, Marburg, Germany) is a novel B domain truncated rFVIII in which the heavy and light chains of FVIII are covalently fused to achieve a single polypeptide protein.[47,48] Following thrombin activation, it is structurally and functionally indistinguishable from endogenously generated FVIIIa. However, it too demonstrates assay discrepancy measuring about 50% lower by one-stage assay compared to chromogenic assay (which is used for its potency assignment). A laboratory field study demonstrated consistent and predictable differences in the assay results across laboratories and FVIII plasma concentrations allowing for a "correction factor" of 2 applied to the one-stage assay results in order to align with chromogenic assay results.[49]

3.2 | Unfolded protein response

The ER, as the site of folding and disulphide bond formation for nascent secreted proteins, is a unique oxidating environment. The UPR is an adaptive signalling pathway that serves to prevent the accumulation of misfolded proteins within the ER and to minimize the stress of oxidative protein folding.[35,50] Chronic unresolved accumulation of unfolded proteins within the ER leads to apoptosis. FVIII has been shown to be prone to misfolding in the ER lumen, inducing ER stress-induced oxidative damage, activation of the UPR and apoptosis. This correlates with reduced FVIII expression in cellular expression systems in vitro as well as reduced plasma expression in vivo with gene transduction, including with viral gene therapy vectors. Several bioengineered variants designed to alter ER chaperone interactions, and improve folding, have exhibited reduced UPR activation, oxidative stress and apoptosis and are associated with improved FVIII expression in vitro and in vivo. A single missense mutation, F309S within a hydrophobic pocket of the FVIII A1 domain, enhances expression twofold[51] and further enhances the expression efficiency of the 226/N6 B domain variant up to 10-fold in a preclinical viral gene transfer study.[32] Given these observations, what evidence is there that FVIII constructs currently being evaluated for clinical gene therapy risk intracellular misfolding and resultant induction of UPR, oxidative stress and apoptosis?

Zolotukhin and colleagues used a codon-optimized BDD-FVIII within an AAV vector to transduce wild-type C57BL/6 mice.[52] Although there was no resultant increase in ALT levels, there was upregulation of UPR sentinel chaperone proteins at increased doses of the AAV vector. Notably, the same was not observed with null vectors or those containing a FIX transgene. Lange et al[53] also observed a delayed and transient cellular stress response in the liver in a haemophilia A mouse model when BDD-FVIII was delivered by AAV and was expressing at supraphysiological levels (>200%). Staber et al[54] showed that a lentiviral vector to deliver BDD-FVIII induced UPR and biochemical markers of ER stress that could be attenuated with a modified B domain that contains 11 engineered N-glycans. Although none of these studies demonstrated clear evidence of cellular toxicity, studies of FVIII expression in platelets have demonstrated that high expression of FVIII can lead to apoptosis.[55]

These animal model results suggest the potential for BDD-FVIII and its codon-optimized variants, to induce UPR and cellular stress within the ongoing clinical trial programmes. Previously noted BMN 270 was investigated within mice and cynomolgus monkeys.[56] BMN 270 produced therapeutic levels of FVIII activity within an immune-incompetent double knockout mouse (FVIII and recombinant activating gene 2 knockouts). Intracellular retention of FVIII was noted within hepatocytes. However, they did not demonstrate any evidence for activation of the UPR when liver homogenates were evaluated 5, 12 and 24 weeks post-BMN 270 dosing. Given the observations in previous studies, these time points could have missed a transient UPR response. Their study indicated that another construct with a stronger promoter did induce expression of molecular chaperones consistent with UPR activation. Although detailed studies to evaluate for UPR activation were not performed in the monkeys, no elevation of plasma ALT or AST levels was observed.

Within the phase 1/2 BMN 270 study,[57] 11 of the 15 subjects exhibited elevations in ALT (13 were Grade 1 and 1 was Grade 2) with 8 having accompanying increases in AST. This included 6 of the 7 subjects at the highest AAV dose (6×10^{13} vg/kg) and 4 of the 6 subjects at the 4×10^{13} vg/kg dose. All of these were assessed as non-serious and of limited duration occurring between 0.4 and 15.4 weeks from the vector administration. No subjects exhibited any evidence for a T cell-mediated immune response associated with the ALT elevations. The subjects in the BMN 270 trial, as has been typical in the other haemophilia programmes, received a course of corticosteroids with the intent to blunt any immune response and salvage protein expression. However, if these ALT increases are a biomarker for, even transient, cellular stress from FVIII expression, corticosteroids may not be the optimal intervention. This may provide an opportunity to explore the impact of bioengineered FVIII variants with improved folding and secretion efficiency and also consideration for the use of systemic antioxidants.[35]

3.3 | Rapid clearance of FVIII from circulation

Although bioengineering has produced EHL-rFIX products with 3- to 5-fold half-life extension, EHL-rFVIII products have been limited to about 1.3- to 1.5-fold that of standard rFVIII.[58] This is likely due to their continued interaction with endogenous VWF where they are subject to clearance as part of the FVIII-VWF complex.[59] Novel bioengineering strategies are attempting to overcome this "ceiling"

effect. A VWF-albumin fusion protein exhibits a significant prolongation of VWF half-life in vivo.[60] However, as long as FVIII can distribute to unmodified endogenous VWF, this strategy would have a limited overall impact on FVIII half-life. However, it is now recognized that the D'D3 fragment of VWF, containing the FVIII-binding region, is sufficient to stabilize FVIII in vivo.[61] The D'D3 fragment can be modified to extend its half-life (targeted glycosylation, albumin/Fc fusion). The challenge remains to increase the affinity of the D'D3-FVIII interaction to reduce redistribution to unmodified VWF in vivo. One strategy to address this is through a covalent linkage between a D'D3/Fc fusion and a rFVIIIFc fusion molecule.[62] The rFVIIIFc fusion molecule was further modified with unstructured hydrophilic, biodegradable polypeptide polymers (XTEN, Amunix, Mountain View, CA, USA). This covalent dimer of FVIII and D'D3 has demonstrated up to fourfold longer half-life compared to unmodified rFVIII in a mouse model.

3.4 | FVIII with reduced immunogenicity

That few bioengineered FVIII molecules have been moved into clinical trials can be attributed in part to the significant immunogenicity of even the native molecule. Strategies to reduce the immunogenicity of FVIII have been thus far elusive. However, with new insights on how FVIII interacts with antigen-presenting cells, several approaches are being investigated. These include the expression of rFVIII within human cell lines[63,64] with the aim of retaining human-specific post-translational modifications, such as its glycosylation profile; porcine-human hybrids which eliminate highly immunogenic peptide sequences[65]; Fc-fusion and PEGylation[58]; targeted mutagenesis of the FVIII C1 and C2 domains[66] or fusion of FVIII with nanobodies with enhanced affinity for VWF,[67] both of which aim to reduce dendritic cell endocytosis.

4 | BIOENGINEERING OF FIX

4.1 | FIX Padua

The first wave of clinical trials for haemophilia B between 1996 and 2006 showed transient expression of FIX with correction of the bleeding phenotype.[13] AAV distinguished itself as having the best safety/efficacy and risk/benefit ratios, and the liver was established as the most suitable target organ for expression. However, the challenge of the host immune response including pre-existing immunity to AAV as well as a cytotoxic immune response to the vector capsid limited the success of these early trials. This led to investment in additional preclinical studies to improve the vectors through investigation of alternative serotypes with improved transduction efficiency, improvements in the transgene and promoters and better understanding of the mechanisms of the immune response. The proof of concept for gene therapy for haemophilia B was achieved between 2010 and 2014. Despite low plasma levels of FIX (~2%-5%), the expression has been durable and has been accompanied by correction of the bleeding phenotype (>90% bleed reduction and liberation from the need for regular FIX prophylaxis).[68] Nevertheless, the continued problem of a cytotoxic response has precluded simply delivering higher doses of vector to target higher plasma levels of FIX.

The current wave of clinical trials for gene therapy is all benefitting from use of a bioengineered FIX construct with higher specific activity. Although these trials have applied some additional improvements in vector design and immunomodulation, the most impactful innovation has been the use of a point mutation (R338L) in FIX. This is a naturally occurring FIX mutation described originally by Simioni et al[69] at the University of Padua, identified within a family with inherited thrombophilia who expressed this form of FIX. These patients demonstrated up to an eightfold higher plasma activity of FIX, all attributable to a higher specific activity conferred by this point mutation. FIX Padua had been investigated in a number of preclinical studies and exhibited a 5- to 10-fold increase in activity in every context regardless of the vector used or target cell. FIX Padua has now been used in 2 clinical trials[70,71] thus far and is to be incorporated into a third. In the study reported by George and colleagues,[70] FIX Padua as part of an AAV-based gene therapy targeting the liver achieved sustained FIX plasma activity of 33.7% + 18.5% (range 14%-81%) across 9 subjects at a vector dose of 5×10^{11} vg/kg. Not only did this level or expression obviate the need for FIX infusions, but it virtually eliminated all spontaneous bleeding events. This represents an important pairing of improvements in gene therapy vector design with the advantages of a bioengineered construct.

4.2 | Bioengineered FIX for subcutaneous delivery

Not all bioengineering strategies with FIX are being applied only to gene therapy constructs. You et al[72] have described a bioengineered rFIX with enhanced biological properties that allows for alternative delivery strategies. Through a rational design approach, they have bioengineered rFIX with resistance to inhibition by antithrombin, increased affinity for FVIIIa and increased catalytic activity. This modified rFIX, CB2679d/ISU304, exhibits 22-fold enhanced potency in an in vitro clotting assay and in vivo via a murine tail clip model and has an eightfold enhanced duration of aPTT activity in vivo compared with wild-type rFIX. These enhanced properties are being evaluated in a clinical trial (NCT03186677, www.clinicaltrials.gov) administered subcutaneously for prophylaxis. The pharmacokinetic profile was evaluated in several cohorts in a crossover, ascending dose design compared to IV standard recombinant FIX confirming the 22-fold greater potency and long mean residence time. This is now being evaluated in a final cohort with daily subcutaneous dosing. Modelling from the preclinical studies suggests that subjects should be able to maintain normal plasma levels of FIX with a daily subcutaneous administration.

5 | CONCLUSIONS

The current wave of recombinant therapeutics for haemophilia has seen the benefits of bioengineering, leading to EHL factor products,

enhanced specific activity to improve the efficacy and safety of gene therapy and introduced the first non-factor therapeutic, a bispecific antibody. Even the current novel products will likely be supplanted through additional bioengineering efforts to further enhance pharmacokinetic profiles, reduce the immunogenicity of factor VIII and even improve on the efficacy of bispecific antibodies as a factor VIIIa-mimetic. The next wave of therapeutics may be influenced from new insights from analyses of next-generation sequencing and transcriptome databases that hope to elucidate new targets for bioengineering.

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DISCLOSURES

The author has no competing interests.

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