Recent advances in developing specific therapies for haemophilia

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Summary

Therapy of haemophilia has undergone very rapid evolution in the past 10 years. The major limitation of current replacement therapy is the short half-life of factors VIII and IX. These half-lives have been extended by addition of various moieties, allowing less frequent infusion regimens. Entirely novel approaches have also entered the clinic including a bispecific antibody that mimics factor VIII and strategies that rebalance the haemostatic mechanism by reducing antithrombin through inhibition of synthesis. These two treatments are available by subcutaneous injection at infrequent intervals and both can be used in patients with neutralising antibodies (inhibitors). Finally, a cure may be on the horizon with preliminary evidence of success for gene therapy in haemophilia B and A.

Key Words: - haemophilia, bleeding disorders, therapy, Factor VIII, Factor IX, gene therapy

Short title: Advances in haemophilia therapies

Introduction

Haemophilia A and B are X-linked, monogenic bleeding disorders due to factor VIII (FVIII) deficiency and factor IX (FIX) deficiency respectively. Approximately 1/5000 males are affected with haemophilia A, with 1/30000 males in haemophilia B (Mannucci & Tuddenham 2001; Bolton-Maggs & Pasi 2003). In its severe form, spontaneous bleeding occurs from a young age, typically in the form of haemarthroses and skeletal muscle haematomas in the absence of antecedent trauma, resulting in chronic arthropathy with significant deformities in the long term if untreated. In the pre-clotting factor concentrate era, death in both conditions was primarily due to intracranial haemorrhage or other life threatening bleeds and patients rarely survived beyond 10 years. Prophylactic administration of factor concentrate to maintain circulating factor levels above 1IU/dL (1%) of normal has resulted in a dramatic reduction in bleeding frequency and its associated complications, with substantial improvements to life expectancy over recent decades (Darby et al. 2007).

In the last forty years, treatment has largely focused on incremental changes progressing from using cryoprecipitate (for haemophilia A) or whole plasma (for haemophilia A or B) to plasma derived and more recently, recombinant specific factor concentrates. Treatment based on cryoprecipitate transfusion was rich in FVIII, and was in routine use until the 1970s (Mannucci 2002). Developments were made to move towards factor concentrates to deliver a more consistent dose compared with cryoprecipitate, as well as significantly improving safety of concentrates through viral inactivation steps by heat, solvent detergent and nanofiltration methods.

Despite significant improvements to ensure safety, there are disadvantages with current clotting factor replacement. Inhibitor formation (neutralising allo-antibodies which form against factor concentrate) causes significant morbidity for patients, which affects 20-45% with severe haemophilia A and 3% of haemophilia B who are previously untreated patients (PUPs) (Astermark et al. 2010, Peyvandi et al. 2016). Immune tolerance induction regimens (ITI) are used to eradicate the inhibitor, but can take several months or years, and are only effective in haemophilia A. While 80% respond to

this treatment with resolution of the inhibitor, ITI is highly expensive as it requires very high doses of concentrate to succeed (50-200U/kg alternate days or daily) (Collins et al. 2013). At least 20% of patients are resistant to ITI with long term consequences for their health due to hitherto only partly successful agents for bypassing FVIII or FIX deficiency in the presence of neutralising antibody. To reduce the risk of inhibitor formation, some haemophilia centres elect to use plasma derived concentrates for previously untreated patients (PUPs) which may have lower inhibitor risks compared with recombinant products. The SIPPET study which recruited children showed that in PUPs the use of recombinant FVIII had an 87% increase in risk of inhibitor formation rates compared with plasma derived products (Peyvandi et al. 2016). However, the use of plasma derived products does not eliminate the risk of viral transmission nor of prions causing variant Creutzfeldt-Jakob Disease.

Pharmacokinetics in each individual varies and therefore standardised dosing may result in patients being undertreated if factor clearance is higher than expected. Routine primary prophylaxis for patients with severe haemophilia A is offered on alternate days in the United Kingdom and for severe haemophilia B every 3-4 days. For haemophilia A, FVIII concentrate dosing is recommended on the basis of a half-life of 8-12 hours (Srivastava et al. 2013), but large patient cohorts studied suggest that FVIII half-life has a much wider range, estimated between 6-25 hours (Björkman et al. 2009; Björkman et al. 2010). Patients with levels which routinely fall below 1U/dL are at risk of increased bleeding (Collins et al. 2009). Variation in personal lifestyles or bleeding phenotypes may mean patients require different trough level targets. To account for these variations, clinicians may elect to do individualised pharmacokinetic studies and the results could reduce costs of treatment with increased frequency of dosing (Collins 2012). Caution in interpreting results is required given challenges with accurate factor level measurement, with differing levels reported between 1-stage and chromogenic assays for standard half-life and extended half-life concentrates (Kitchen et al. 2014).

Factor concentrates remain highly expensive even with national contract negotiation (O'mahony et al. 2015) and stretch national health budgets, while being unaffordable in less economically developed countries. Prophylactic treatment to reduce mortality and improve quality of life is therefore beyond the means of many haemophilia patients across the world. Support for haemophilia patients in the form of regular medical review**s** is also lacking in many under resourced areas.

The last decade of research in haemophilia has yielded many potentially life-changing therapies with exciting novel treatments such as RNA silencing agents (siRNA), FVIII mimetics and gene therapy which are currently in clinical trials and show great promise. There remains a significant unmet medical need to improve self-care, reduce requirements for venous access and frequency of dosing, reduce or eliminate inhibitor risk and ultimately devise a cure for this condition. In this review, we summarise recent advances in haemophilia treatment and current clinical progress made in these areas.

Factor concentrates

Half-life extension has been an important evolution in concentrate manufacturing to enable patients to reduce the frequency of dosing with better maintenance of factor levels, especially higher trough levels. This has been more successful in haemophilia B than in haemophilia A (where the half-life is dominated by that of its carrier – von Willebrand Factor). Table 1 lists recently licensed FVIII and FIX concentrates or those with completed clinical trials. Historically, standard half-life FVIII concentrates were first to be commercially available, followed by FIX products but in recent years extended half-life concentrates (EHLs) for haemophilia B have been developed more quickly, with clinically significant increases over standard half-life FIX concentrates of 2.4-4.8 fold (Young & Mahlangu 2016). Previously published trial data on EHLs has been collated into a review for clinicians to choose an option that would be best suited for their patients as per recommendations issued by the United Kingdom Haemophilia Centres' Doctors' Organisation (Collins et al. 2016).

Name of concentrate		Company	Mechanism	Cell line	Half life (h)	Current status	Reference
FVIII – EHLs							
Elocta (rFVIII-Fc)	*Efraloctocog alfa*	*Biogen/Sobi*	*Fc (IgG1) fusion to B-domain deleted FVIII*	*HEK*	*19*	*Licensed*	(Mahlangu et al. 2014)
Adynovi (BAX855)	Octocog alfa pegol	Shire	Pegylated to full length FVIII, porcine sequence	CHO	14.3	Licensed	(Konkle et al. 2015)
N8-GP	Turoctocog alfa pegol	Novo Nordisk	Pegylated (40kDa) B-domain truncated FVIII	CHO	19	In Phase 3 trials	(Lentz et al. 2013)
BAY94-9027	Damoctocog alfa pegol	Bayer	Pegylated (60kDa) B-domain deleted FVIII	BHK	18.7	In Phase 2/3 trials	(Boggio et al. 2014)
FVIII – standard half-life concentrates							
Nuwiq	*Simoctocog alfa*	*Octapharma*	*B-domain deleted FVIII*	*HEK*	*15.1*	*Licensed*	(Lissitchkov et al. 2017)
NovoEight	Turoctocog alfa	Novo Nordisk	B-domain truncated FVIII	CHO	11.2	Licensed	(Jiménez Yuste et al. 2015)
Kovaltry/Ibli as	BAY81-8973	Bayer/CSL Behring	Full length FVIII	BHK	13.4	Licensed	(Shah et al. 2015)
FIX - EHLs							
Alprolix	*rFIX-Fc*	*Biogen/Sobi*	*Fc fusion to FIX*	*HEK*	*82.1*	*Licensed*	*(Powell et al. 2013)*
Idelvion	rFIX-FP	CSL Behring	Albumin fusion to FIX	CHO	102	Licensed	(Santagostino et al. 2016)
N9-GP	Nonacog beta pegol	Novo Nordisk	Pegylation of FIX	CHO	96.3	Licensed	(Collins, Young, et al. 2014)

Table 1 – list of new FVIII/FIX standard and EHL concentrates. Italics denote fourth generation concentrates.

FIX concentrates

EHL FIX concentrates have enabled dosing frequency in haemophilia B to be reduced from approximately twice weekly to once every 7-14 days (Collins et al. 2016). Three FIX products have completed Phase 3 clinical trials in haemophilia B patients, with each using different methods to prolong circulating FIX levels. rFIX-Fc (Alprolix, Biogen) fuses the Fc immunoglobulin region with FIX, while rFIX-FP (Idelvion, CSL Behring) combines FIX with albumin, and nonacog beta pegol (N9-GP, Novo Nordisk) is a pegylated version of FIX. Despite the differing products from a manufacturing perspective, haemostatic efficacy was reported at similar levels, with all three products requiring 1-2 injections for bleed resolution and an overall efficacy of between 96.7-97.2%. Median annual

bleeding rates (ABR) were estimated at between 0-3.0 for all three products. (Santagostino et al. 2016; Collins, Young, et al. 2014; Young & Mahlangu 2016).

FVIII concentrates

FVIII EHLs have yielded less progress with half-life prolongation of 1.5 times compared with standard FVIII concentrates (Mahlangu et al. 2014; Young & Mahlangu 2016). As with FIX EHLs, Fc fusion or pegylation has been used to produce FVIII EHLs, with half-lives of new products ranging from 14.3-19 hours. Because of this modest extension in half-life, subject to pharmacokinetic analysis for individualised dosing, it is recommended to suggest a reduction in frequency of dosing from alternate days to once every 3-4 days (Collins et al. 2016). The relative lack of improvement in half life in FVIII concentrates are thought to be related to the interaction of FVIII with vWF. In a few patients, no increase in the interval dosing is seen when an EHL is used compared with a standard product (Makris 2012; Tang et al. 2013; Croteau & Neufeld 2015).

Manufacturers have continued to produce new versions of recombinant standard half-life concentrates with putative improvements in their manufacturing process. Table 2 lists the different generations of products and their characteristics. Increasingly, the trend has been towards eliminating human or animal plasma derived proteins in large scale manufacturing to reduce, as far as possible, risks of as yet unidentified viruses to ensure the highest level of safety.

	Cell line	Human/animal protein supplementation in culture media	Albumin as stabiliser	Products
First generation	Animal (CHO/BHK)	Yes	Yes	Recombinate, Kogenate, Helixate
Second generation	Animal (CHO/BHK)	Yes	No	Refacto, Kogenate FS, Helixate NexGen
Third generation	Animal (CHO/BHK)	No	No	Advate, Refacto AF, NovoEight, Kovaltry
Fourth generation	Human (HEK)	No	No	Nuwiq

Table 2. Standard half-life recombinant FVIII concentrates

Two new, third generation concentrates have been produced: BAY81-8973 (Kovaltry, Bayer), a full length FVIII, is based on the same sequence as octocog alfa, a second generation concentrate (Kogenate FS, Bayer) but with the removal of human or animal proteins in its manufacture apart from the use of a baby hamster kidney (BHK) cell line (Keating 2016). Turoctocog alfa (NovoEight, Novo Nordisk) is a B-domain truncated FVIII, with the B domain specifically designed to accommodate pegylation. The sequence in this FVIII product has been used to develop a pegylated FVIII, Turoctocog alfa pegol (N8-GP, Novo Nordisk) as an EHL.

Fourth generation concentrates have also been in clinical trials with the aim of reducing inhibitor formation. This differs from third generation concentrates by using human instead of animal cell lines for production, with further potential to reduce immunogenicity. Simoctocog alfa (Nuwiq, Octapharma) is currently the only fourth generation, standard half-life concentrate produced with human embryonic kidney (HEK) cells and post-translational modifications are designed to be similar to plasma derived FVIII. A multicentre, open-label Phase 3 trial in 32 adults in a standard prophylaxis regime for ≥6 months who were severe previously treated patients (PTPs) showed mean ABRs of

2.28 for all bleeds, 1.16 for spontaneous bleeds and 1.00 for traumatic bleeds. Biochemical in vitro analysis showed normal restoration of thrombin generation, and no discrepancy between 1-stage APTT based and chromogenic assays. A further phase 3 study is still under recruitment for PUPs (Liesner et al. 2016), however participation rate had not yet reached the stage of 50 EDs to inform on inhibitor rates compared with other currently licensed products.

Non-clotting factor based therapies

Alternative approaches using methods beyond clotting factor replacements have been developed, primarily with the aim of restoring haemostasis through bypassing the deficient coagulation protein. Table 3 summarises the approaches, of which two methods aim to inhibit proteins that are themselves inhibitors of the coagulation pathways, thus targeting both haemophilia A and B patients (a monoclonal anti-TFPI antibody and a small interfering RNA (siRNA) molecule to reduce antithrombin levels), and the use of an antibody to mimic the activity of FVIII as a co-enzyme for haemophilia A. These alternative approaches have efficacy in patients with inhibitors who do not respond to conventional clotting factor replacement. These newer agents also are amenable to the simpler mode of delivery by subcutaneous injection, eliminating the need for intravenous administration.

Class	Products available	Mechanism of action	Advantages	Disadvantages
Antithrombin inhibition	Fitusiran	siRNA against antithrombin	Infrequent, monthly subcutaneous dosing Suitable for patients with FVIII inhibitors Effective in both haemophilia A and B	Variable recovery of antithrombin levels in washout period post injection One fatal thrombosis reported in use of fitusiran with FVIII concentrate used for breakthrough bleeding – product currently suspended (September 2017)
Monoclonal antibodies against TFPI	Concizumab	Binding of K domain of TFPI, resulting in inactivation	Subcutaneous dosing for easier administration Suitable for patients with FVIII inhibitors Effective in both haemophilia A and B	Early evidence of non-linear pharmacokinetics in animal models
FVIII-mimetic	Emicizumab	IgG4 antibody with FVIII coenzyme activity	Once weekly, subcutaneous injection Suitable for patients with FVIII inhibitors	Not suitable for patients with Haemophilia B Early trials have shown adverse events in combination with activated protein C concentrates

Table 3. Non-clotting factor based therapies and their mechanisms.

Antithrombin targeting

A strategy to rebalance the haemostatic system in patients with haemophilia, who have defective procoagulant pathways, works to reduce their natural inhibitor of thrombin generation. Antithrombin (AT) is a serine proteinase inhibitor in the serpin family, and acts in the coagulation system by primarily inhibiting thrombin, Factor Xa (FXa) and Factor IXa (FIXa) through irreversible neutralisation. AT is a key protein in the haemostatic pathway for regulating normal haemostasis, as AT deficiency either in congenital or acquired forms is the highest risk factor for thromboembolic disease (Menache et al. 1992).

Small interfering RNA sequences (siRNA) have been used to target AT by binding with AT mRNA produced by the hepatocyte. Fitusiran is a double stranded RNA molecule which inhibits and degrades AT mRNA and in turn reduces AT levels. As AT inhibits FXa, the reduction in AT potentiates the effect of the procoagulant pathway to restore normal haemostasis. A phase I trial targeting healthy volunteers injected 45ug/kg doses weekly for three weeks and noted a trough AT level at 28 days with an increase in thrombin generation by 334% (Sorensen et al. 2014).

A completed phase I dose-escalation study (NCT02035605) was performed looking at 4 healthy volunteers and 25 patients with moderate or severe haemophilia A or B without inhibitors, at varying doses of administration (healthy volunteers received 0.03mg/kg or placebo, while haemophilia participants received 0.015-0.075mg/kg in weekly doses or 0.225-1.8mg/kg or 80mg in monthly dosing). Initial trial results were promising with mean maximum antithrombin reduction of 70-89% from baseline, with a fall in mean ABR (at all doses) from 9.7 to 5.4 at 29-56 days post last dose. Increase in thrombin generation correlated with decreases in antithrombin level, with median peak thrombin levels at the lower end of the range observed in healthy volunteers, for haemophilia participants who had >75% AT reduction from baseline. No serious adverse events were recorded (Pasi et al. 2017). However, in a Phase 1/2 extension study (NCT02554773) a fatal thrombosis was announced by Alnylam in a press release in September 2017. The patient in question while on fitusiran had received three doses of FVIII concentrate therapy (31-46IU/kg each dose) over three days for exercise induced hip pain when he developed a headache after the third dose. The headache was judged to be secondary to a subarachnoid haemorrhage based on CT imaging, and the patient continued to receive FVIII concentrate 2-3 times daily with deterioration in his clinical status until death from cerebral oedema. Further analysis suggested that the precipitating event for the intracranial bleed was because of a cerebral venous sinus thrombosis. All further trials with fitusiran have been suspended while safety investigations are in progress.

TFPI inhibition

Tissue factor pathway inhibitor (TFPI) is a multivalent, Kunitz-type proteinase inhibitor (KPI) with three KPI domains, and functions by inhibiting generation of FXa and the activity of the TF/FVIIa complex (Broze & Girard 2012). Inhibition of TFPI downregulates the negative feedback loop that regulates tissue factor (TF)/FVIIa–mediated Factor X (FX) activation during the initiation phase of coagulation. Blockage of TFPI inhibition aims to restore TF/FVIIa-mediated FXa generation that is sufficient for haemostasis.

Concizumab (mAb 2021), a monoclonal antibody was designed to inhibit KPI domain 2 on TFPI which is known to bind FXa. In a study by Hilden et al. investigating in vitro and in vivo effects, the addition of mAb 2021 to haemophilic plasma and whole blood shortened clot times in a dilute PT assay and improved thrombin generation. In a transient rabbit haemophilia model induced by FVIII antibodies, mAb 2021 was given at doses from 0.5-8mg/kg. Bleeding volumes for doses $\geq$1mg/kg reduced bleeding compared with rabbit haemophilia controls, and were not significantly different from normal rabbit controls. At doses of 10mg/kg, subcutaneous injection in rabbits was also shown to be effective (Hilden et al. 2012).

A human phase 1 dose finding study was conducted in both healthy volunteers (n=28) and haemophilia patients (n=24) with concizumab injected both intravenously and subcutaneously. Three adverse events were reported in those receiving concizumab, one episode of superficial thrombophlebitis which resolved spontaneously and one of transient proteinuria and another of

abdominal pain. No clinically relevant changes in platelets, PT, aPTT, fibrinogen, or antithrombin were found. Non-linear pharmacokinetics were noted and a dose-dependent procoagulant effect of concizumab was seen as increased levels of D-dimers and prothrombin fragment 1 + 2. No cases of thrombosis were observed (Chowdary et al. 2015). In a separate ex-vivo spiking study, platelet poor plasma from haemophilia patients was spiked with concizumab. At doses of 10nM, thrombin generation approached near those of healthy controls (Waters et al. 2017). Two Phase 2 trials are yet to open investigating the effect of concizumab on haemophilic patients with and without inhibitors (registered trials NCT03196284 and NCT03196297 respectively).

FVIII co-enzyme mimetics

Emicizumab, currently in Phase 3 trials, is a humanised, IgG4 antibody designed to have the same functional characteristics as activated FVIII (FVIIIa). The antibody functions in a bispecific format by binding FIXa to one arm of the antibody and FX to the other, accelerating the activation of the FX zymogen to FXa, thus propagating further thrombin generation. An antibody which closely mimics the activity of FVIIIa, can bypass any inhibitors to FVIII, and carries a further significant advantage as delivery is by subcutaneous injection rather than intravenous infusion.

A Phase I trial with an initial loading dose of 1mg/kg, followed by one of 0.3mg/kg, 1mg/kg or 3mg/kg weekly for 12 weeks was carried out in severe haemophilia A patients with and without inhibitors. In 18 patients (6 at each dose), all dosing levels showed a reduction in median ABR from 32.5 to 4.4, 18.3 to 0, and 15.2 to 0 respectively. In 8 of 11 patients with inhibitors, no bleeding occurred, with an accompanying reduction in use of clotting factors to stop bleeding and no identified antibodies to emicizumab (Shima et al. 2016).

Phase 3 trials with emicizumab have commenced and are continuing. HAVEN 1 has completed recruitment (n=109) in patients with inhibitors 12 years and older. Patients were divided into three arms (emicizumab, no emicizumab and emicizumab with previous history of bypassing agent prophylaxis). 63% of patients on emicizumab reported no bleeds on the trial, with an overall reduction in bleed rate of 87% compared with the no emicizumab arm, with accompanying improvements in health status and quality of life assessments ($P<0.001$). Those who had prophylactic bypassing agents previously and now on emicizumab prophylaxis had a bleeding rate that fell by 79%.

15% of patients receiving the drug reported injection site reactions. Five patients developed major adverse events during the Phase 3 trial, all with concomitant use of activated protein complex concentrate (aPCC) to stop active bleeds (two episodes of thrombosis and three incidents of thrombotic microangiopathy). One death occurred from the five patients with a major AE reported but was judged to be unrelated to emicizumab administration, and two of these five patients successfully restarted emicizumab with no further side effects (Oldenburg et al. 2017). Preliminary work on characterising the thrombogenicity of emicizumab have started with ROTEM analysis on citrated, whole blood samples of haemophilia patients. Ex-vivo spiking of emicizumab and a bypassing agent (either rFVIIa or aPCC) significantly decrease the clot time (CT) and clot formation time (CFT) compared with emicizumab alone, with aPCC showing greater shortening of CT and CFT compared with rFVIIa (Yada et al. 2017). This may underline concerns that an unregulated FVIII mimic which functions in a permanently active state produces a deregulated and intrinsically unstable thrombin generation system, with more investigation required on the causes of thrombogenicity. Additional data suggest that emicizumab lacks the ability to function in the

absence of phospholipid binding and has a much lower binding affinity compared with FVIII to FIXa and FX (Kitazawa et al. 2017) which may compensate for the permanently 'switched on' activity. Subsequent trial protocol amendments have prohibited the use of aPCC with emicizumab to prevent further serious adverse events.

More phase 3 trials are underway, with HAVEN 2 (NCT02795767) targeting inhibitor patients aged less than 12 years old, with further studies to investigate non-inhibitor patients (HAVEN 3, NCT02847637) and reductions in dosing frequency to fortnightly or monthly (HAVEN 4, NCT03020160).

Gene therapy

A one-time intravenous infusion to treat haemophilia permanently is a paradigm-shifting therapy for patients. Current treatment, because of its expense and pharmacodynamics, has meant that prophylactic factor concentrate treatment had only the modest goal of keeping trough levels of factor just above 1IU/dL to stop major acute bleeding and its chronic sequelae. Fairly recent data, by the simple but effective means of studying patients with mild haemophilia, clearly demonstrated that bleeding in joints is only completely eliminated at levels of 15IU/dL or more (Uijl et al. 2010).

Even in its extended half life forms, clotting factor replacement (as described above) requires repeated intravenous infusions, although injections are required less frequently. Replacement factor therapy necessitates interval dosing which comes with peaks and troughs of factor levels; when these trough levels are insufficiently high this results in occasional breakthrough bleeds.

Both haemophilia A and B are ideal candidates for gene therapy; a small increase in factor levels can substantially ameliorate the bleeding phenotype. Gene therapy has had a resurgence of interest for its curative potential in many conditions but previously had its setbacks; initial trials in 2002 in severe combined immunodeficiency successfully treated the condition, but the use of an oncoretroviral vector inevitably led to insertion of the transgene into the host genome, which in a tiny proportion of insertions upstream of the LMO2 gene in some recipients caused a leukaemia like syndrome (Thomas et al. 2003). Safety has therefore been a key concern of subsequent trials since.

In haemophilia, adeno-associated virus (AAV) is the vector of choice, selected for its lack of pathogenicity in humans in that the majority of viral DNA is not integrated into the host genome and remains episomal, thus reducing likelihood of oncogenesis and germ line transmission. Currently, there remain hypothetical risks to tumourigenesis with AAV gene therapy despite an absence of oncogenic events in over 130 AAV gene therapy studies. Low level of integration has been observed with wild-type AAV viruses, estimated at 0.1% of virus transduction for chromosome 19 specific integration, a site of known predilection for AAV integration (Huser et al. 2002). More recent findings have identified clonal integration of AAV2 in hepatocellular carcinoma (HCC) samples in 11 out of 193 cases studied, which have upregulated cancer driver genes (*CCNA2, TERT, CCNE1, TNFSF10, KMT2B*) (Nault et al. 2015). Notably among the 11 AAV-positive biopsies, 5 biopsies presented with other well-known HCC-driving conditions (such as alcohol consumption or hepatitis B or C infections) and 4 biopsies presented with AAV-unrelated mutations which have been previously linked with liver cancer development (such as *TP53* or *AXIN1*).

Current participants recruited to trials have been limited to adult populations for whom the liver is fully developed, such that effective hepatocyte transduction is not lost in mitotically active and growing liver tissue. Patients identified as suitable for AAV gene therapy must be seronegative for

antibodies to the specific capsid used. Anti-AAV antibodies across all serotypes are estimated at 10-80%, but some serotypes such as AAV5 (10-20%) and AAV8 (10-30%) have less population immunity compared with AAV2 (up to 70%) which has historically been the most frequently studied (Halbert et al. 2006, Calcedo et al. 2009, Mingozzi et al. 2013), and thus switching capsids may provide a solution to increasing access for patients to gene therapy. A summary of current trials conducted in haemophilia A and B are in Table 4 below.

	Company	Vector	Planned enrolment number	Dose injected, vg/kg (number of patients receiving dose)	Factor variant	Trial status	Outcomes	Reference/Trial number
FVIII								
BMN270	BioMarin	AAV5	15	6e12 (1), 2e13 (1), 6e13 (7), 4e13 (6)	B-domain deleted FVIII (Refacto sequence)	Open, recruitment complete	Mean peak 116-129IU/dL at 6e13vg/kg dose with durable response at >52 weeks	(Pasi et al. 2017) NCT02576795
GO-8	UCL/St Jude	AAV8	18		B-domain truncated, FVIII V3 variant	Recruiting	Early phase recruitment – data awaited	NCT03001830
Spk-8011	Spark Therapeutics	Novel capsid based on AAV8	30	5e11 (2) 1e12 (1)	B-domain deleted FVIII	Recruiting	2 subjects treated at 5e11 had early response levels at 11% and 14%	NCT03003533
SB-525	Sangamo Biosciences	AAV2/6	20		B-domain deleted FVIII (Refacto sequence)	Recruiting	Early phase recruitment – data awaited	NCT03061201
FIX								
scAAV2/8-LP1-FIXco	UCL/St Jude	AAV2/8	16	2e11 (2), 6e11 (2), 2e12 (6+2), 6e12 (2)	LP1 promoter, FIXco	Open, recruitment complete	Sustained FIX:C of 1-6IU/dL in first 10 patients treated and reported.	(Nathwani et al. 2011, 2014) NCT00979238
AskBio009	Shire	AAV8	30		TTR promot	Closed	8 patients treated with 7 losing expression,	NCT01687608

(BAX335)					er, FIX Padua		1 maintaining FIX:C at 20IU/dL at 2.5 years	
Spk-9001	Spark Therapeutics/ Pfizer	Novel capsid	15	5e11	FIX Padua	Recruiting	10 patients treated with mean sustained FIX:C of 29IU/dL	(George et al. 2017) NCT02484092
AMT-060	uniQure	AAV5	10	5e12 (5) 2e13 (5)	LP1 promoter, FIXco	Recruiting	10 patients treated, mean FIX:C (n=5, low dose) = 5.2IU/dL, (n=5, high dose) = 6.9IU/dL at 18 months of follow up. 1 patient did not achieve increase in levels; remaining 8 of 9 treated patients discontinued prophylaxis	(Miesbach et al. 2017) NCT02396342
DTX101	Dimension Therapeutics	AAVrh10	12		wtFIX	Closed	6 patients treated, no data published, study terminated in May 2017	NCT02618915
SB-FIX	Sangamo Biosciences	AAV2/6	12		Zinc finger nuclease gene editing	Recruiting	Early phase recruitment – data awaited	NCT02695160

Table 4. Summary of gene therapy trials in Haemophilia A and B.

Haemophilia B

The first successful study in severe haemophilia B patients showed sustained FIX expression in a cohort of 10 patients, while studies prior to this used a variety of vectors resulting in only transient increases in patients' factor levels before returning to baseline (Nathwani et al. 2011; Nathwani et al. 2014; Lheriteau et al. 2015). To overcome difficulties observed in previous trials, the FIX gene was codon optimised, such that the codon sequence of each amino acid was modified to reflect sequences of human proteins that were highly expressed in the human liver. In addition, a FIX self-complementary sequence enabled one virion to have both the coding and complementary sequence such that the transgene can become readily transcriptionally active. Finally, a pseudotyped vector was used with an AAV2 genome but with the AAV8 capsid, which has increased liver tropism and reduced antibody seroprevalence in humans compared with other serotypes of AAV (Sands 2011; Boutin et al. 2010).

Patients were enrolled to this Phase I study at low, intermediate and high vector doses, producing sustained FIX:C expression at 1–6IU/dL in all ten subjects during a follow-up period extending to a median of 6 years, concomitant with decrease in FIX concentrate usage by 90%. The most promising current trial sponsored by Spark Therapeutics and Pfizer have used a novel engineered AAV capsid, with a FIX Padua transgene which is known to demonstrate 8-fold higher activity and demonstrates a thrombophilic tendency in patients with otherwise normal coagulation states, compared with wild-

type FIX (Simioni et al. 2009). In 10 patients, mean FIX:C levels of 29IU/dL were achieved in those reaching 12 weeks of follow-up (George et al. 2016).

Haemophilia A

Although haemophilia A is 5 times more prevalent than haemophilia B and therefore the more obvious candidate for gene therapy, the cDNA size of the FVIII gene has been a hurdle to developing an expression cassette that is packageable into an AAV vector for FVIII production. The FVIII full length cDNA is 7 kilobases (kb) long compared with the FIX cDNA of 1.5kb; and a B-domain deleted form of FVIII cDNA is 4.4kb. Typically, AAV can package up to 4.6-5kb, which leaves little room for regulatory elements of the virus (Lheriteau et al. 2015). Sequences greater than 5kb in size have been used successfully but in vivo potency of the transgene was reduced by 2-3 fold (Dong et al. 2008; Chen et al. 2009).

With additional technical challenges to overcome, clinical trials in haemophilia A have thus lagged those of haemophilia B. The transgene used has been either a B-domain deleted or truncated FVIII to reduce expression cassette size, with adjustments in the regulatory sequences to further minimise total size. Four trials are currently in the recruitment phase using different capsids and promoters (see Table 4). BMN270 (BioMarin) has conducted the first trial with an AAV5 capsid containing a B domain deleted FVIII sequence (SQ). Phase I recruitment has been completed. 15 patients were treated at doses ranging from 6e12 vector genomes (vg)/kg to 6e13vg/kg. Patients that received the highest dose of 6e13vg/kg (n=7) achieved mean FVIII:C levels of 116-129IU/dL with durable responses lasting greater than 52 weeks. A subsequent slightly lower dose 4e13vg/kg cohort (n=6) reached 31-42IU/dL at 12 weeks. Mean ABR changed from 17 to 0 for patients previously on prophylaxis, with mean annualised FVIII infusion rates falling from 139 to 0. 7. Subjects have all had a grade 1 transient elevation in ALT which was resolved with corticosteroids (Pasi et al. 2017). Three other trials now recruiting are using different capsid and FVIII variations.

Therapeutic monitoring strategies

There are existing challenges in current methods of factor level monitoring post concentrate treatment, which can vary significantly depending on the concentrate and assay used, with inter-laboratory results showing considerable differences (CVs of 15-26% with chromogenic assays and 12-19% in one stage assays) (Kitchen et al. 2016). In gene therapy, transgenes with engineered *F8* or *F9* may present similar issues, as secreted FVIII or FIX may differ from currently available concentrates. EHLs also pose a challenge with FIX EHLs showing inter-laboratory variation of up to 161% against the International Standard defined by the Scientific and Standardization Committee (SSC), while one-stage FVIII:C and FIX:C results with EHLs are heavily dependent on the type of assays used (Kitchen et al. 2014).

As patients transition to non-clotting factor therapies, treatment monitoring and establishing 'equivalence' to FVIII:C or FIX:C will become an increasing problem, as uncertainty surrounds the issue of how clinicians determine adequate prophylaxis aside from a bleeding history assessment. In FVIIIa mimetics, considerable discrepancy exists between the measurement of their activity by 1 stage clotting assays, chromogenic assays and thrombin generation (Goodman et al. 2017), even as FVIII:C monitoring by chromogenic assay shows a linear relationship with emicizumab concentrations (Adamkewicz et al. 2017). As anti-TFPI and anti-AT therapies work on a very different part of the coagulation cascade, correlation with a patient's past treatment modalities will be even more complex. Monitoring patients on different therapies will require novel strategies and standardisation.

Clinical laboratories will need to adapt to new methods; only then can there be informed assessment of breakthrough bleeding at patient reviews.

Future therapies

Innovative strategies which have not yet reached the stage of clinical trials revolve largely around three areas.

Firstly, in haemophilia A, attempts are being made to extend the half-life of FVIII by improving FVIII-vWF interaction. Previous work has shown that expression or infusion of the D'D3 region of vWF is sufficient to stabilise and prolong half-life of FVIII in vWF knockout mice, with a fusion D'D3 vWF-Fc protein demonstrating significantly longer half-life than a D'D3 synthetic vWF fragment alone (Yee et al. 2014). By making use of the FVIII-vWF interaction, strategies may be designed to co-infuse recombinant vWF, or FVIII variants with stronger affinity to vWF. Pestel et al. have used recombinant D'D3-albumin fusion protein and co-infused this with FVIII-SingleChain (CSL Behring) into haemophilia knockout mice, rats and rabbits which improved the half-life of FVIII to at least 1.5-3 fold (Pestel et al. 2017). Kawecki et al. used a different approach by developing a bivalent nanobody which binds both vWF and albumin which extends half-life of vWF by 8-fold and FVIII by 6-fold at 7 days in wild type C57Bl6 mice (Kawecki et al. 2017).

Secondly, work on non-clotting factor replacements to haemophilia have continued. Alternative FVIIIa mimetics are in development, with new alternative binding antibodies similar to emicizumab showing evidence of FXa generation (Leksa et al. 2017). With the success of antithrombin targeting therapy in clinical trials, Ayme et al. have created anti-AT nanobodies with high binding affinity, which in a haemophilia knockout murine model have blocked AT activity by 50-100% and normalised thrombin generation. The potential of nanobodies with their short transgenic sequences could be used in AAV gene therapy approaches to permanently lower antithrombin levels (Ayme et al. 2017). Polderijk et al. have alternatively targeted a different aspect of the anticoagulant pathway by using a modified α1-antitrypsin to inhibit activated protein C which showed restoration of thrombin generation in three separate mouse models (Polderdijk et al. 2017).

Thirdly, modifications to clotting factors to produce hyperfunctional variants may reduce frequency of intravenous injection or enable subcutaneous administration instead. A variant of FIX termed CB2679d was developed by Levy et al. with increased affinity for FVIIIa and resistance to AT inhibition, resulting in 20-fold potency increase in vitro and in a haemophilia B dog model in comparison with wild type FIX (Levy et al. 2017).

Conclusion

Treatment for haemophilia in the coming years is entering a revolutionary phase, with the advent of multiple treatments coming to market such that factor replacement soon will no longer be the sole option. Approved extended half-life factor concentrates have started to improve the quality of life of patients through reducing injection frequency. Emerging non-factor therapies in trial have potential to alter significantly the management of patients in the medium term, as clinicians and patients now face the prospect of having many more choices for treating haemophilia and tailoring it to needs and lifestyle of individual patients, with each of the therapies targeting different unmet needs in the haemophilia population. These treatments already significantly change the management of patients with inhibitors and offer more convenient choices for non-inhibitor patients with subcutaneous

injections on a less frequent basis. Gene therapy offers potential for cure by means of a single treatment. Furthermore, in resource limited settings where access to healthcare and factor concentrates is inconsistent, it has obvious advantages. However, it will undoubtedly incur a high cost for initial treatment.

Despite the number of novel therapies in the haemophilia field, standard half-life factor concentrate will remain as a mainstay of treatment over the next years given that these are familiar to patients and have extensive safety data, predictability and ease of monitoring efficacy by using factor assays. For most patients, these treatments are highly effective and they may not feel compelled to alter their treatment to the newer products.

Overall, the emergence of agents with novel modes of action now in Phase 3 trials shows that new treatments are coming ever closer to marketing approval, bringing with them the prospect of highly individualised care in haemophilia as part of the treatment conversation to achieve optimal management that patients and their clinicians wish to achieve.

Author contributions

GL and EGDT wrote and critically reviewed the manuscript. ACN provided additional content expertise and critically revised the manuscript.

Declarations

GL is a consultant to Freeline Therapeutics Ltd; ACN is Chief Scientific Officer of Freeline Therapeutics Ltd. EGDT is a consultant to Biomarin Pharmaceutical Inc. and Freeline Therapeutics Ltd.

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