

## Theme Issue Article

# Recombinant clotting factors

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### Summary

The recombinant era for haemophilia began in the early 1980s with the cloning and subsequent expression of functional proteins for both factors VIII and IX. Efficient production of recombinant clotting factors in mammalian cell culture systems required overcoming significant challenges due to the complex post-translational modifications that were integral to their pro-coagulant function. The quick development and commercialization of recombinant clotting factors was, in part, facilitated by the catastrophic impact of viral contamination of plasma-derived clotting factor concentrates at the time. Since their transition into the clinic, the recombinant versions of both factor VIII and IX have proven to be remarkable facsimiles of their plasma-derived counterparts. The broad adoption of recombinant ther-

apy throughout the developed world has significantly increased the supply of clotting factor concentrates and helped advance aggressive therapeutic interventions such as prophylaxis. The development of recombinant VIIa was a further advance bringing a recombinant option to haemophilia patients with inhibitors. Recombinant DNA technology remains the platform to address ongoing challenges in haemophilia care such as reducing the costs of therapy, increasing the availability to the developing world, and improving the functional properties of these proteins. In turn, the ongoing development of new recombinant clotting factor concentrates is providing alternatives for patients with other inherited bleeding disorders.

### Keywords

Recombinant, factor VIII, factor IX, factor VIIa, von Willebrand factor

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## The development of recombinant factor VIII (rFVIII)

### It was the best of times and the worst of times

In the early 1970s, biochemist Herbert W. Boyer and geneticist Stanley Cohen pioneered a new scientific field called recombinant DNA technology (1). A venture capitalist, Robert A. Swanson, excited by the prospects for the technology, joined with Dr. Boyer and founded Genentech in San Francisco on April 7, 1976 (2). In a few short years Genentech successfully produced the first human recombinant protein (somatostatin) with *Escherichia coli* (3). Genentech scientists then cloned human insulin and growth hormone, and human insulin became the first recombinant DNA product to be marketed in 1982 (4). As Genentech grew, it attracted new scientists set on conquering a new challenge, the cloning and expression of FVIII.

Meanwhile, in 1981, two Harvard scientists, Mark Ptashne and Thomas Maniatis, convinced four venture capitalists to help start a new firm with the goal of using genetic engineering to create protein-based substances for use in medical therapies (5). Upon the addition of five additional scientists, including three postdoctoral students from Massachusetts Institute of Technology, Genetics Institute began its research. One of the company's first efforts was to clone and produce FVIII.

The "race to the gene" has been personally recounted by Jane Gitschier and reveals the underlying motivations at the time (6). Certainly the intellectual challenges and potential commercialization opportunities were important. However, early 1982 saw the first reports of what would become the catastrophic impact of human immunodeficiency virus (HIV) on the haemophilia community (7). Within two years of this report, it is estimated that 63% of the 15,500 patients living with haemophilia in the United States contracted HIV from plasma-derived clotting factor con-

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centrates (8). Thus, the plight of haemophilia patients became a driving force. A recombinant, virus-free replacement product was the goal. In December 1983, Genetics Institute reported that it had isolated and cloned part of the gene for FVIII. On April 20, 1984, scientists at Genentech reported publicly the cloning of the entire FVIII sequence and the first laboratory production of recombinant FVIII protein. The virtually simultaneous efforts of Genentech and Genetics Institute were presented at the World Federation of Haemophilia Congress in Rio De Janeiro in August 1984 and finally published in *Nature* in November, 1984 (9–12).

### Overcoming new challenges

Though the world's attention was on FVIII, factor IX (FIX) was actually the first of the hereditary bleeding disorder proteins to be cloned, in 1982 (13), and the challenges of recombinant clotting protein expression were already recognized. The complexities of FVIII and FIX would require that they be produced in a mammalian cell expression system. Insights from the study of their structure and function had demonstrated that they required extensive post-translational modifications. Two cell systems, Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells, had proven to be efficient expression systems. Both were capable of high-level expression, produced post-translational modifications, including complex carbohydrates, similar to human proteins, and were adaptable to large-scale 'fermentation' techniques to facilitate commercial production (14). Experience with CHO cell lines had demonstrated other important advantages (15, 16): they exhibited relatively low proteolytic activity, which was important for the production of intact FVIII protein; they were resistant to infection by all but a few human viruses, an important safety concern during development of the recombinant clotting factors; and dihydrofolate reductase (DHFR) deficient strains had been developed enabling amplification of genes of interest through selective cell culture medium containing methotrexate (17).

The first experiences in expressing recombinant FIX (rFIX) were limited. However, the application of DHFR-amplification technology within CHO cell lines led to a 100-fold increase in the efficiency of rFIX expression, levels 30-fold higher than within human plasma (18).

Recombinant FVIII (rFVIII) expression would prove to be more difficult. Bovine serum was typically added to the media of mammalian cell lines to facilitate efficient growth and protein expression. However, to facilitate commercialization, eliminating the need for serum was essential to reduce costs, reduce exposure to animal viruses and to ease purification. Whereas rFIX-producing cells were amenable to adaptation to serum-free medium, rFVIII was not expressed under these conditions (15). It was determined that FVIII had a high affinity for the phospholipid membrane. Thus, rather than being liberated into the medium upon secretion, it remained associated with the membrane of the CHO cells and was subsequently degraded. The addition of von Willebrand factor (vWF) to serum-free CHO cell media led to expression levels similar to those observed with 10% bovine serum-supplemented media (19). The cloning of vWF in 1985 (20–23) would have an immediate impact on rFVIII expression. An expression system was then designed in which CHO cells engineered to express rFVIII were sub-

sequently transfected with a human vWF. This cell line, now expressing both rFVIII as well as recombinant vWF (rvWF), was amenable to adaptation to serum-free medium and allowed for a foundational production cell line to facilitate the industrial process (15).

The fermentation process to produce large quantities of rFVIII has had 20 years of optimization. The basic principles (16) have been similar amongst all the commercially produced recombinant clotting factors: a master cell bank is archived, working cell banks (derived from the master) are then grown slowly in successively larger flasks and eventually inoculate stainless steel bioreactors of several thousand liters. Upon achieving optimal cell culture density, the media undergoes a series of purification steps that can include physical restriction, immunoaffinity (murine monoclonal antibody) and ion-exchange chromatography, pasteurization, solvent-detergent or even nanofiltration steps with some recent commercial preparations. This removes all medium components, host cell residues and even rvWF and a volume reduction from as high as 7,000 liters to as little as 1 liter of highly purified rFVIII. Bovine or human albumin was a necessary stabilizer for rFVIII with initial production technology. The final finishing steps result in vial filling and lyophilization.

### The road to the clinic

The biopharmaceuticals overcame major technical hurdles to produce rFVIII, one of the largest and most complex proteins produced by recombinant DNA technology. The industry scientists in this area were innovating production optimization for consistent large scale production and spawning an entire industry of biochemical engineering (24). The third hurdle was one of regulatory uncertainty. The regulatory agencies had not yet produced a 'road map' toward approval for recombinant proteins. There were concerns about the potential impact of host cell DNA and host cell proteins on the health of individuals receiving recombinant products. Much effort was exerted to demonstrate the consistency of the final recombinant material from batch to batch, and including sterility and quality control testing it would not be unusual for 400–600 individual tests to be performed on every lot of recombinant factor produced (16, 25). Baxter's Hyland laboratories collaborated with Genetics Institute on their rFVIII project, whereas, the Genentech rFVIII program had been acquired by Bayer's Miles Lab/Cutter Biological, leading to the "first generation" of commercial rFVIII, Recombinate<sup>®</sup> and Kogenate<sup>®</sup>, respectively.

Recombinant FVIII would prove to have excellent efficacy and similar pharmacokinetics to plasma-derived (pd)FVIII in preclinical studies. However, upon initiating the clinical trials, there was significant concern with regard to the immunogenicity of the rFVIII as compared to its plasma-derived counterparts (26) and potential immune responses to rodent proteins by virtue of the cell culture and murine monoclonal antibody columns used during the production process (27, 28). Murine and rabbit immunogenicity studies had suggested that rFVIII and pdFVIII were structurally and immunologically similar (14). These concerns would only be addressed in the clinic.

White et al. reported the clinical experience with the first two patients to receive rFVIII (Recombine) in the *New England*

**Table 1: Factor VIII therapies.**

FVIII therapy	FVIII molecule	Human or animal-derived protein exposure	Virus screening, removal, and inactivation methods
Plasma-derived	Full-length	Yes	Donor screening, MAbs purification, heat treatments (dry, in solution), NAT testing, IA, IE, S/D
Recombinant			
First-generation rAHF	Full-length	Yes	IA, IE
Second-generation KFS, RF	B-domain-deleted or Full-length	Yes	IA, IE, S/D, ultra- or nanofiltration
Third-generation rAHF-PFM	Full-length	No	IA, IE, S/D

rAHF=Recombinate<sup>®</sup>; KFS=Kogenate<sup>®</sup> FS; RF=ReFacto<sup>®</sup>; rAHF-PFM=Advate<sup>®</sup>;  
 IA=immunoaffinity chromatography; IE=ion-exchange chromatography; MAbs=monoclonal antibody;  
 NAT=nucleic acid testing; S/D=solvent/detergent.

*Journal of Medicine* in 1989 (29). The two reported men, aged 39 and 18, were engaged in a pharmacokinetic study followed by home therapy for up to 12 months. The first infusion occurred on March 27, 1987 at the University of North Carolina Hospital in Chapel Hill. That this occurred less than three years after the cloning of the FVIII gene remains a marvel of the recombinant era. Recombinate was eventually available in the USA from December 1992 and Kogenate early the following year.

### Further bioengineering and processing advances

Very soon after the initial mammalian expression studies were performed, it was demonstrated that the B domain of FVIII was dispensable for its procoagulant activity. Bioengineered variants could be generated in which most or all of the B domain (roughly 38% of the primary cDNA sequence) could be removed from the rFVIII construct with a resultant increase in the yield of FVIII (30). A B-domain-deleted (BDD)-rFVIII, r-VIII SQ, was originally characterized by Pharmacia & Upjohn (Stockholm, Sweden) and clinical studies began in 1993. This first of the bioengineered forms of FVIII to come to the clinic had some potential advantages as it was a homogeneous protein product, exhibited increased specific activity and it was stable in final formulation in the absence of added human serum albumin (31). However, there were some biochemical differences that were observed. A FVIII potency assay discrepancy exists in which one-stage clotting assays of BDD-rFVIII activity, using commercial activated partial thromboplastin time (APTT) reagents, are consistently about 50% lower than that measured by the chromogenic assay (a two-stage assay) (32, 33). This assay discrepancy occurs *in vitro* as well as *ex vivo* after analysis of plasma from treated patients. This is an unexpected observation following deletion of the B domain, and the mechanism has not yet been explained. However, the discrepancy can be abrogated by using

mixtures of source phospholipid in which the content of phosphatidylserine is maintained below 10% (33). A smaller degree of one-stage/two-stage potency discrepancy has also been observed for other rFVIII products when measured against pdFVIII standards (34). r-VIII SQ was eventually developed commercially through an agreement with Genetics Institute, then a subsidiary of American Home Products (and eventually Wyeth), in 1987 and was eventually approved as ReFacto<sup>®</sup> in March 2000.

Despite the perceived increased safety afforded by rFVIII preparations over pdFVIII, advances in plasma screening, better characterization of source plasma and significant breakthroughs in viral inactivation technology made safety advantages a theoretical discussion. However, due to the uncertainty that has loomed with regard to as yet unknown pathogens and the potential impact of prion disease on the haemophilia population reliant on blood derivatives (35), there was a further push to eliminate all human and animal protein additives from rFVIII preparations. Additional changes included the addition of viral inactivation steps (solvent/detergent) to the manufacturing process. At first glance, this appears dichotomous for a recombinant preparation; however, by this time viral inactivation had become a mainstay of the regulatory approval process. The resultant “second generation” and “third generation” products are compared in Table 1.

### rFVIII clinical studies

Over the past 20 years, a wealth of clinical information has been amassed for rFVIII in patients with severe or moderately-severe haemophilia A. The published clinical trials for the currently available rFVIII therapies are presented in Table 2. These include the “first-generation” therapy, Recombinate<sup>®</sup> [Antihæmophilic Factor (Recombinant)] (rAHF); the “second-generation” therapies, Kogenate<sup>®</sup> FS Antihæmophilic Factor (Recombinant) (KFS) and ReFacto<sup>®</sup> Antihæmophilic Factor (Recombinant) (RF) (a B-domain-deleted rFVIII); and a “third-generation” therapy, Advate<sup>®</sup> [Antihæmophilic Factor (Recombinant), Plasma/Albumin-Free Method] (rAHF-PFM). To date, there have been no published head-to-head trials of these commonly prescribed therapies for haemophilia.

To assess the clinical response to therapy, two populations of patients with haemophilia are normally studied: previously untreated patients (PUPs) and previously treated patients (PTPs). Factor replacement regimens are evaluated in patients receiving either therapy for prophylaxis of bleeding episodes (including surgery) or on-demand therapy for acute bleeding episodes. Efficacy, as determined by achievement of haemostasis, is evaluated in some studies using a four-point rating scale. “Excellent” is defined as arrest of bleeding with abrupt pain relief within eight hours (h); “Good” is defined as pain relief or reduction of bleeding that was delayed or required more than 1 infusion; “Fair” is defined as a probable or slight beneficial response; “None” is no response. In many trials, patients are monitored for years.

### Haemostatic efficacy

Results from multicenter clinical studies in the PTPs clearly indicate that haemostatic efficacy is achievable in a high percentage of patients on the rFVIII therapies studied (Table 2)

Table 2: Trials evaluating rFVIII therapy in previously treated patients and previously untreated patients.

Recombinate
<b>White and colleagues, 1997 (36)</b>
<b>Study design:</b> P, OL, MC trial in patients with moderate or severe haemophilia A who were previously treated with FVIII therapy (n = 69)
<b>Treatment:</b> <b>Part 1:</b> pdFVIII 50 IU/kg, followed 1 week later by rAHF 50 IU/kg to determine half-life and recovery at preinfusion, 10–15 minutes, 1, 3, 6, 9, 12 and 24 hr post-infusion, then <b>Part 2:</b> rFVIII given as prophylaxis and on demand, dosing regimens empiric and at discretion of investigator and patients based on severity and nature of bleeding episode, and response to treatment
<b>Outcomes:</b> <b>Mean in vivo half-life (±SD)(Part 1):</b> pdFVIII 14.7 ± 5.1 hr vs. rFVIII 14.7 ± 4.9 hr; <b>Mean half-life of rFVIII over time (Part 2):</b> statistically significant trend (P = .015) reflecting longer mean half-lives observed at months 18 and 24, mean values at all other timepoints were NS from baseline; <b>Mean incremental recovery* (±SD) (Part 1):</b> pdFVIII 2.47 ± 0.33 vs. rFVIII 2.40 ± 0.97 (P = .59); <b>Mean incremental recovery of rFVIII over time (Part 2):</b> no statistically significant trend observed over a 30-month time interval (P = .70); <b>Response to rFVIII treatment:</b> rated as “good” or “excellent” in 91.2% (3195/3481) bleeding episodes; <b>ADR rFVIII:</b> 0.096% (13/13,591 infusions), primarily infusion related and nonserious; no inhibitor development to rFVIII was observed
<b>Bray and colleagues, 1994 (40)</b>
<b>Study design:</b> P, OL study of rFVIII in PUPs with moderate or severe haemophilia A (n = 79)
<b>Treatment:</b> rFVIII on demand for acute bleeding episode or for prevention of bleeding, dosing regimen individualized based on patient presentation
<b>Outcomes:</b> A total of 1785 infusions were administered to treat 810 evaluable bleeding episodes; median number of exposure days for assessable study patients was 11; 92% of bleeding episodes responded as expected to 1 or 2 rFVIII infusions; actual and predicted rFVIII recoveries correlated well in patients who had at least 1 recovery determination (n = 65), indicating consistent potency of the product from lot to lot; <b>ADRs:</b> 2 nonrecurring (erythematous rash), one possibly related to infusion, and one likely unrelated to infusion; <b>Inhibitor development to rFVIII:</b> 17 (23.9%) subjects developed inhibitors: 5 with peak titers >10 BU, 12 with peak titers ≤10 BU (range 0.5–10); inhibitors disappeared in 5 (29.4%) patients upon retesting 2 to 16 months after the last inhibitor assay
<b>Comments:</b> Inhibitor risk associated with rFVIII seen in this study is comparable to PUPs treated with pdFVIII
Advate
<b>Tarantino and colleagues, 2004 (37)</b>
<b>Study design:</b> Patients had to have moderate or severe haemophilia (FVIII ≤2%), age >10 years, weight >35 kg, and prior treatment with FVIII concentrates for ≥150 exposure days; the study was carried out in 3 parts: <b>Part 1:</b> DB, R, CO pharmacokinetic comparison of rAHF-PFM and rAHF <b>Part 2:</b> OL, UC, prophylactic treatment regimen for evaluation of efficacy, safety, and immunogenicity <b>Part 3:</b> DB, R, CO study to compare the pharmacokinetic profile of rAHF-PFM manufactured at 2 different sites, processed as pilot-scale and full-scale, respectively; study patients were randomized to either Part 1 and Part 2 (n = 56) or Part 2 and Part 3 (n = 55)
<b>Treatment:</b> <b>Pharmacokinetic studies:</b> first dose of factor therapy was 50 ± 5 IU/kg, second dose to be identical to the first, with a washout of 72 hr, but not more than 4 weeks between doses <b>Prophylactic treatment:</b> 25–40 IU/kg QOD or TIW, but doses >40 IU/kg were allowed for particular situations (e.g., expected increased physical activity) at the discretion of the investigator and in accordance with the institution's standard of care
<b>Outcomes:</b> <b>Part 1:</b> pharmacokinetic profiles demonstrated bioequivalence between rAHF-PFM and rAHF based on AUC (1534 ± 436 IU·h dL <sup>-1</sup> vs. 1530 ± 380 IU·h dL <sup>-1</sup> ), half-life (12.0 ± 4.3 hr vs. 11.2 ± 2.5 hr), and recovery* (2.4 ± 0.5 vs. 2.6 ± 0.5) <b>Part 2: Efficacy:</b> there were 510 bleeding episodes evaluated; of these, 473 (93%) were managed with 1 or 2 infusions of rAHF-PFM and 439 (86%) had efficacy ratings of “excellent” or “good”; <b>ADR:</b> no serious product-related ADRs and 19 (0.15%, based on ratio of 19 ADRs and 12, 597 infusions) nonserious product-related ADRs; the 19 ADRs included, but were not limited to, taste perversion, headache, dizziness, hot flashes, and diarrhea; overall, rAHF-PFM was well tolerated; <b>Inhibitor development:</b> 1 subject developed a low titer, nonpersistent inhibitor (2.0 BU) after 26 exposure days <b>Part 3:</b> pharmacokinetic profiles demonstrated bioequivalence between the pilot-scale and full-scale rAHF-PFM formulations based on AUC (1511 ± 416 IU·h dL <sup>-1</sup> vs. 1466 ± 409 IU·h dL <sup>-1</sup> ), mean adjusted recovery* (2.6 ± 0.5 vs. 2.4 ± 0.5), and half-life (11.7 ± 1.9 hr vs. 11.6 ± 2.1 hr).
ReFacto
<b>Lusher and colleagues, 2003 (38)</b>
<b>Study design:</b> OL, MC, LT study conducted to determine haemostatic efficacy of RF in 3 separate study populations: <b>PTPs:</b> patients with severe haemophilia (FVIII concentration <2 U/dL), at least 7 years old, and have received at least 30 exposure days to FVIII per year (n = 113) <b>PUPs:</b> patients with severe haemophilia A, no previous exposure to FVIII therapy, and documented vaccination against HBV (n = 101) <b>Surgery patients:</b> patients with moderate or severe haemophilia A (FVIII concentration of 2–5 U/dL), and a medical need for major surgery estimated to require daily treatment with FVIII concentrate for at least a week (n = 42) All enrolled patients were being treated at an HTC
<b>Treatment:</b> RF dosing based on patient presentation; for <b>surgery patients undergoing major surgery</b> , the bolus dose was given pre-op to attain a target FVIII concentration of 0.5–1.0 IU/mL, and for <b>surgery patients undergoing minor surgery</b> , target FVIII concentration was 0.2–0.5 IU/mL
<b>Outcomes:</b> <b>PTPs:</b> Total number of exposure days: 43, 507 (median = 313); <b>Efficacy:</b> 10,594 bleeding episodes; 73% effectively treated with 1 infusion of RF; 97%–99% of all infusions rated as “good” or “excellent” efficacy; <b>ADR:</b> 101 minor ADRs reported in 35 subjects that had possible or probable relation to RF (0.22%, 46,318 infusions); <b>De novo inhibitor development:</b> 1 subject after 113 exposure days

Table 2: Continued

**PUPs:** Total number of exposure days: 29,050 (median = 197); **Efficacy:** 31 average bleeding episodes per patient; 65% bleeding episodes resolved after 1 infusion; 92% of all infusions rated as “excellent” or “good” efficacy by investigators, and 92%–95% rated as “excellent” or “good” by study subjects; **ADR:** 35 adverse events reported with possible or probable relation to RF treatment (0.11%, 30,637 infusions), most were considered mild or moderate; however, 4 cases were considered severe, including a case of fever, a catheter port infection, an anaphylactic reaction, and an FVIII antibody increase; **De novo inhibitor development:** 32 subjects developed inhibitors after a median duration of 12 exposure days (range, 3–49); inhibitors disappeared in 43% and 75% of the high-level inhibitor and low-level inhibitor subjects, respectively, after undergoing ITI

**Surgery patients:** Median exposure days: 20; **Efficacy:** 99.6% of all infusions rated as “excellent” or “good” by the surgeon and treating physician; **ADR:** 68 adverse events in 23 patients were reported; of these events 5 in 2 patients were probably related to RF, and 3 in 2 patients were possibly related to RF

#### Kogenate FS

##### Abshire and colleagues, 2000 (39)

**Study design:** Males 12 to 60 years old with severe haemophilia A (stage 1, plasma FVIII <1%; stages 2/3, plasma FVIII <2%) and previously treated with rFVIII, pdFVIII, or cryoprecipitate; **Stage 1:** OL, R, CO pharmacokinetic and safety trial evaluating bioequivalence of KFS to rFVIII<sup>†</sup> (n = 35)

**Stages 2/3:** OL to examine safety and efficacy of KFS (n = 71)

**Treatment:** **Stage 1:** no study drug for first 5 days (NA) or 4 days (EU), then KFS or rFVIII 50 IU/kg

**Stages 2/3:** KFS 20 IU/kg TIW in initial period (NA: 4 weeks; EU: 2 weeks), then patients returned to prestudy treatment of on demand or prophylaxis

**Outcomes:** **Stage 1:** pharmacokinetic profile was comparable between KFS and rFVIII; **Stages 2/3:** 11,687 cumulative total exposure days, 12,456 infusions; **Efficacy:** 2585 evaluable bleeds, 93.5% resolved with 1 or 2 infusions; 80.5% responses rated as “excellent” or “good”; **ADRs:** 0.27% of infusions were associated with ADRs and were mild or moderate, except 1 patient who experienced CP and palpitations that resolved after treatment with analgesics; most common ADRs were injection-site reaction, rash, rash with pruritus, sweating, and taste perversion; 24 of these ADRs were described as at least remotely related to study drug;

**Inhibitor development:** none observed

#### Data on file (42)

**Study design:** LT, P trial in patients with severe haemophilia A (<2% FVIII activity) who were previously untreated or minimally treated (N = 61) with FVIII therapy

**Treatment:** KFS, dosing based on patient presentation

**Outcomes:** Total number of evaluable bleeds were 1173; 89% of bleeding episodes treated effectively with 1 or 2 infusions of KFS; **Inhibitor development:** 16%

**Comments:** Inhibitor development consistent with that seen in PUPs treated with pdFVIII and rFVIII; **Limitations:** study unpublished, study ongoing

\*IU dL<sup>-1</sup> per IU kg<sup>-1</sup>. †Kogenate®. ADR=adverse drug reaction; AUC=area under the curve; BU=Bethesda units; CO=crossover; CP=chest pain; CS=cross-sectional; DB=double-blind; EU=European Union; FVIII=factor VIII; HBV=hepatitis B virus; HTC=haemophilia treatment center; ITI=immune tolerance induction therapy; KFS=Kogenate® FS; LT=long-term; MC=multicenter; NA=North America; NS=not significant; OL=open-label; P=prospective; pdFVIII=plasma-derived FVIII; PTPs=previously treated patients; PUPs=previously untreated patients; QOD=every other day; R=randomized; rAHF=RECOMBINATE; rAHF-PFM=ADVATE; RF=ReFacto®; rFVIII=recombinant FVIII; SD=standard deviation; TIW=three times weekly; UC=uncontrolled.

(36–39). It must be emphasized that these studies are not directly comparable because of differences in study design (number of patients, criteria for patient enrollment, etc) and other factors (numbers of evaluable bleeds and exposure days, etc). Overall, however, a “Good” to “Excellent” response has been reported in 80.5% to 93% of patients for each of the four therapies. Furthermore, a high percentage of resolution of bleeding has been achieved; in three studies, the percentage of bleeding episodes that resolved with one or two infusions ranged from 89% to 93%.

Haemostatic efficacy has also been evaluated in PUPs (40–42). Clinical studies with rAHF-PFM are ongoing in this population, but data on rAHF, RF, and KFS are available. As in the PTP trials, these studies are not directly comparable because of different study designs and other factors. Nevertheless, resolution of approximately 90% of bleeding episodes occurred after one to two infusions with each of the therapies tested.

### Inhibitors

The incidence of inhibitors in PTPs is generally low. For example, in 108 PTPs treated with rAHF-PFM in a phase 2/3 pivotal study, the frequency of inhibitors was less than 1% (37). These antibodies were generally low titer and non-detectable after eight weeks and non-recurring. In the pivotal rAHF trial, none of the 65 patients developed inhibitors after 30 months

(36). In clinical studies with KFS, no FVIII inhibitors were reported in 71 patients after a mean of 54 exposure days (39). The incidence of inhibitor development is similar with RF; only one in 113 PTPs treated with RF developed a high titer inhibitor after 113 exposure days (38). After 18 months of continued therapy, however, this patient’s inhibitor level continued to rise and the patient was taken off treatment.

Nevertheless, the question of whether rFVIII exhibits a higher risk for inhibitors in PUPs is an ongoing controversy (43, 44). Wight and Paisley (45) reviewed the cumulative incidence of inhibitors from studies of multiple pdFVIII, single pdFVIII and rFVIII products. In PUPs treated with a single pdFVIII, the cumulative risk of inhibitor development was 0–12%, whereas it was 36–39% in those treated with a rFVIII. This comparison is hampered, however, by comparing small, non-prospective, even single-center experiences with pdFVIII, with prospective, carefully monitored safety and efficacy studies. These comparisons also do not take into account many genetic and non-genetic risk factors for inhibitor formation in PUPs that have recently been elucidated.

Three comparative historical cohort studies have been published in the last year, which are providing some focus to the question of product-related risk of inhibitors in PUPs: The French cohort (46), the CANAL study (47) and the UK cohort (48). These studies were most recently the subject of a detailed

discussion at the World Federation of Haemophilia Global Forum in 2007 (49). The French study included a cohort of patients ( $n=62$ ) treated with a single pdFVIII (Facteur VIII LFB) (50) and a cohort of patients ( $n=86$ ) treated with rFVIII (Kogenate or Recombinate) (51). The two cohorts were compared, taking into account potential genetic (FVIII mutation, ethnicity, family history) and environmental (age at first infusion) risk factors. They observed an adjusted relative risk (aRR) of 2.4 (95% CI:1.0–5.8,  $p=0.049$ ) for all inhibitors among those treated with rFVIII compared to the single pdFVIII. For high inhibitors ( $>5$  BU), the aRR was 2.6 for the rFVIII cohort but this did not reach statistical significance (95% CI:0.7–9.6,  $p=0.157$ ). Notably, however, the rFVIII cohort included 20% non-caucasians compared to 11% in the pdFVIII cohort. Not all patients in this study had at least 50 exposure days. This study also did not include intensity of treatment and the effect of prophylaxis in the multivariate analysis. The CANAL (Concerted Action on Neutralizing Antibodies in severe haemophilia A) study, is a multicenter, retrospective cohort study investigating 366 consecutive PUPs born between 1999–2000. In this study, 23 pdFVIII products and three different rFVIII products (Recombine, Kogenate and ReFacto) were included. The multivariate analysis included molecular defect, ethnicity, age at first exposure, intensity of treatment and prophylaxis but did not include family history. They observed a RR of 0.8 (95% CI:0.5–1.3,  $p=0.34$ ) for all inhibitors when comparing those treated with pdFVIII with rFVIII and a RR of 0.9 (95% CI:0.8–2.5,  $p=0.72$ ) for high titer inhibitors. They also observed no increased risk for inhibitors in patients switching between FVIII products including those that switched between pdFVIII and rFVIII. This is also in keeping with other studies in PTPs in which patients who switched from pdFVIII to rFVIII products exhibited no increased risk for inhibitors compared to those that remained on pdFVIII (43, 52–54). Finally, the UK cohort included 348 PUPs, also treated with a variety of products, 132 with pdFVIII, 172 with rFVIII. The multivariate analysis included molecular defect, ethnicity, family history of inhibitors, and age at first exposure but did not include intensity of treatment and prophylaxis. They observed an odds ratio (multivariate analysis) of 1.8 for all inhibitors related to rFVIII product therapy (95% CI:0.9–3.7). Taken together, there is a trend to higher inhibitor risk for the rFVIII products, though two of the three cohort studies failed to reach statistical significance. However, there are significant potential confounders in the analyses of product-related risk factors for inhibitor development. This leaves one with a rather unsatisfactory resolution to this controversy that may only be addressed by a prospective, randomized controlled trial. One such trial has recently been proposed (Study on Inhibitors in Plasma-Product Exposed Toddlers, University of Milan). Goudemand et al. have suggested that cohort studies that included 200–500 PUPs would be sufficient to demonstrate an intensity of risk of 2 provided the patients could be followed for at least 50 exposure days (55).

Underlying this controversy is also the question of why there should be a difference in inhibitor risk between pdFVIII and rFVIII (56)? Several factors have been proposed including the presence or absence of vWF (57), and the presence of cytokines including TGF $\beta$  (58, 59) in plasma-derived products. Interestingly, the CANAL study actually showed that there was no pro-

tective effect from products that contained higher amounts of vWF compared to those with lower vWF content or compared to rFVIII. Lin et al. demonstrated that commercial rFVIII concentrates contain a significantly higher (~20%) FVIII:Ag content per IU of FVIII than pdFVIII concentrates (60). In addition, they found a significant fraction of rFVIII molecules that do not bind vWF *in vitro*. They could find no differences in FVIII heavy and light chain association or proteolytic fragments between the concentrates to account for this. There is currently no clinical correlate for these *in-vitro* observations. This remains an area ripe for continued investigation.

## Recombinant factor IX (rFIX)

The development of rFIX presented unique challenges for recombinant technology. Though significantly smaller than FVIII at only 415 amino acids and secreted as a single-chain molecule, it also undergoes complex post-translational modifications, many of which are critical to its biochemical and pharmacokinetic properties (61). Amongst all of these post-translational modifications, 12 glutamic acid residues near the amino terminus of FIX are gamma-carboxylated by the hepatic microsomal enzyme, vitamin K-dependent gamma-glutamyl carboxylase. This is required for the interaction of FIX with phospholipid surfaces and for optimal FIX activity. In addition, an amino-terminus propeptide sequence, serves as a recognition signal for gamma-glutamyl carboxylase and following gamma-carboxylation is cleaved off by a Golgi apparatus serine protease known as Paired basic Amino acid Cleaving Enzyme (PACE/Furin) (62). The requirement for gamma-carboxylation and proteolytic processing by furin for the normal function of FIX was a new requirement compared to other proteins produced by recombinant technology.

As previously mentioned, the expression of FIX in amplified CHO cell systems was relatively efficient, achieving concentrations in the medium of up to 180  $\mu\text{g/ml}$  (18). However, the amount of active, fully carboxylated FIX in the medium was only 1.5  $\mu\text{g/ml}$ . Thus the amplified FIX expression had saturated the available carboxylation activity as well as the proteolytic activity of furin. Similar to the story with rFVIII, furin was cloned and co-expressed within FIX-expressing CHO cells resulting in a several fold increase in proteolytic processing of rFIX (62). This, together with the addition of vitamin K to the cell culture, allowed efficient expression of rFIX with high specific activity (270 U/mg, similar to plasma-derived FIX) suitable for commercial development (14). Detailed analysis of rFIX indicated that only 60% of the molecules are gamma-carboxylated at all 12 glutamic acid residues; 35% and 5% of rFIX is gamma-carboxylated at 11 or 10 residues respectively. However, subfractionation of each of these forms indicates that they all exhibit normal procoagulant activity (63, 64). Two additional post-translational modifications may have important biological implications. FIX undergoes sulfation of tyrosine 155 and phosphorylation of serine 158. In rFIX, less than 15% of tyrosine 155 is sulfated and there is negligible phosphorylation of serine 158. Pharmacokinetic studies in subjects with haemophilia B showed that rFIX has 30% reduced recovery in plasma after infusion compared to pdFIX, however plasma half-life was unchanged (61).

FIX-expressing CHO cell lines grow well in the absence of animal or human-derived protein additives, the purification process does not utilize a monoclonal antibody column, and no human albumin or other human proteins are required for stability in the final formulation. Thus rFIX was the earliest of the “advanced generation” recombinant products.

Genetics Institute completed the commercialization of rFIX gaining regulatory approval for BeneFIX<sup>®</sup> in 1998. The recovery difference observed for BeneFIX required a modification of dosing. Nevertheless, the clinical trials in haemophilia B PTPs and PUPS showed that BeneFIX is safe and effective in the treatment of all types of bleeding with patients reporting good (84%) or excellent (82%) response to a single infusion (65). In addition, surgical procedures were rated as excellent or good in all patients treated with BeneFIX. Inhibitors were limited to a single patient among 44 PTPs. The titer remained low (<1 BU) and resolved over 11 months. The pediatric PUP trial demonstrated similar pharmacokinetics as observed in the PTP trial with 94% of bleed responses rated as excellent or good and 90% of bleeding episodes managed with one or two infusions (66). Of the 63 PUP subjects, two (3%) developed high-titer inhibitors (>5 BU), similar to previous studies with pdFIX (67, 68).

## Recombinant factor VIIa (rFVIIa)

By the 1980's, the Danish company, Nordisk, a large supplier of the world's insulin, had also started production of plasma-derived preparations of FVIII and FIX. They eventually merged with Novo in 1989 as Novo Nordisk to become the world's largest supplier of insulin. Dr. Ulla Hedner, a researcher at Novo Nordisk and Professor at the University of Lund in Sweden, had conceived the idea that led to the development of rFVIIa (69, 70). There had been ongoing concern with regard to the thromboembolic events associated with the use of activated prothrombin complex concentrates (APCCs) in inhibitor patients. These concentrates contain both zymogens and activated coagulation proteins, including FVII and FVIIa. Dog models had demonstrated that co-infusion of antithrombin and heparin abrogated the systemic coagulation activation observed with APCC's. Since FVIIa was not inhibited by these agents, FVIIa was purified and infused into the same dog models with no activation of the coagulation system. The potential use of FVIIa as a haemostatic agent was a new concept. FVIIa was purified from plasma and infused into two subjects with haemophilia A and inhibitors, achieving haemostasis (71). It was proposed that pharmacologic doses of VIIa interacted with tissue factor at the site of injury, promoting activation of factor X and in turn generating thrombin.

However, the prospect of purifying FVIIa from plasma, a tedious process, accompanied by the pathogen risks associated with plasma at that time, pushed the research toward recombinant DNA technology. The Novo Nordisk research teams had already begun experiments on recombinant insulin production. Factor VII was originally cloned from cDNA libraries that have been prepared from human liver and Hep G2 cells (72, 73). Recombinant FVIIa was expressed in BHK cells and found to be biochemically very similar to plasma-derived VIIa. The secreted VII protein was identical in amino acid sequence. However, similar

to rFIX, rVII was under-gamma-carboxylated compared to pdFVII exhibiting 50% of molecules gamma-carboxylated at all 10 potential glutamic acid residues, whereas the remaining 50% had nine residues gamma-carboxylated (14). This has no impact on the specific activity. During purification, the secreted rFVII is autoactivated via a cleavage event at Arg152-Ile153, just as occurs for FVII in plasma. Recombinant FVIIa's safety and efficacy was established in preclinical animal models and brought to the clinic as NovoSeven<sup>®</sup>.

Beginning in 1988, a Compassionate Use Programme evaluated bleeding episodes in patients with haemophilia and inhibitors when other therapeutic options had been exhausted or when essential surgery was required and there were no alternative treatments available (74). The experience within this program led to insights on dosing and interval and proved efficacy for joint, muscle, central nervous system bleeding and surgical haemostasis. A Home Treatment Trial was initiated in 1994 and NovoSeven was eventually approved in Europe in 1996 and in the USA in 1999. Approval was extended to acquired haemophilia in 2006.

Although the initial indications for use suggested a dosing range of 90–120 ug/kg/dose every 2–3h, higher doses of NovoSeven, as high as 270–400 µg/kg, have been utilized by some clinicians with apparently increased efficacy (75). Such “high-dose” NovoSeven is believed to produce a “thrombin burst” on the surface of activated platelets leading to activation of FIX and FX in a tissue factor-independent mechanism (76). However, a prospective randomized trial of standard and high-dose NovoSeven for management of hemarthroses showed equivalence, but the reduced number of infusions required with higher dosing was a potential advantage (77). A recent multi-national randomized cross-over clinical trial (FENOC) compared standard dose NovoSeven to 85 U/kg of FEIBA<sup>®</sup>, a commercial activated prothrombin complex concentrate (APCC) (78). Efficacy results were essentially equivalent.

Clinical monitoring has remained elusive for NovoSeven, similar to the problem with patients receiving APCCs. Although the PT invariably shortens after administration, this does not correlate with haemostatic efficacy. There are ongoing investigations in this area. The issue of safety of NovoSeven in broader use has been questioned due to reports of some thromboembolic events (79). However, these have been rare events among haemophilia patients with inhibitors and do not appear to be higher than those occurring with APCCs (80).

In recent years, the use of NovoSeven has expanded beyond its haemophilia and inhibitors indication. NovoSeven has now demonstrated efficacy in the management of patients with congenital FVII deficiency and Glanzmann's Thrombasthenia. Phase II trials continue for trauma-related bleeding, intracerebral haemorrhage, traumatic brain injury and even correction of warfarin-induced coagulopathy (see review [81]).

## Unfulfilled promises

The recombinant era promised clotting factor replacement products that would be safer than their plasma-derived counterparts, consistent manufacturing and processing liberated from the uncertainties of securing source plasma, a potentially unlimited supply

that could drive down costs of replacement therapy, an increase in the utilization of prophylaxis, and wider availability of replacement products for patients in developing countries (82–84).

However, recombinant products have failed to realize some of these goals. Increasing sophistication in plasma testing and viral inactivation have led to an outstanding safety profile for the currently available plasma-derived clotting factor concentrates, though this too at great cost. rFVIII remains approximately 1,000-fold less efficiently expressed in mammalian cell culture compared to other less complicated proteins such as monoclonal antibodies. Although the fermentation and purification process can be completed in approximately two weeks, the extensive quality assurance, control testing and accompanying documentation can take more than six months (25). All of these factors can contribute to the costs of replacement therapy. The European Haemophilia Economic Study Group found that the mean annual direct medical costs per patient in an on-demand therapy group ranged from 24–92,000 euros and between 112–182,000 euros for a prophylactic group (85). In the recently published Joint Outcome Study, the estimated annual cost for prophylactic treatment of one patient with recombinant rFVIII was \$300,000 USD (86). Certainly the supply of FVIII replacement products has increased significantly in the past 15 years. Worldwide demand for FVIII has increased from approximately 2 billion units to almost 5 billion units in 2004 (87). Over 40% of the FVIII infused is rFVIII; however, almost 90% of it is sold in North America and Europe. Increased plasma fractionation throughput has led to a 60% increase in pdFVIII as well over the past 20 years providing an alternative for those without access to recombinant products. There have been significant improvements in utilization in South America, the Middle East, Asia and the Pacific. Even the prospect of an uninterrupted supply of factor replacement products in the recombinant era has been challenged. In 2001, supplies of one widely prescribed rFVIII therapy were reduced for approximately four months due to the detection of bacterial byproducts during processing (88). Although the processing problem was remedied quickly, normal distribution was limited for some time afterward with impacts on continued prophylaxis and elective surgeries.

## Recombinant DNA technology – Still the key to the future?

Despite some unfulfilled promises from the recombinant era, recombinant clotting factors have been enthusiastically embraced by the developed world and are now the preferred therapeutic initiated in new pediatric patients (89–92). Recombinant DNA technology has now become the platform to address remaining challenges facing those with congenital bleeding disorders. Despite 20 years as an expression partner with rFVIII, recombinant vWF may prove to be a viable therapeutic product for von Willebrand disease and enhance future haemophilia therapies. New recombinant clotting factors for other rare bleeding disorders (e.g. FXIII) are now in the pipeline. The lessons learned from recombinant protein expression have impacted approaches to gene therapy and provided a platform for protein bioengineering to produce replacement products with enhanced function, opening up alternative delivery strategies. Enhanced

protein expression systems and transgenic animal technology may lead to more cost-effective manufacturing. A few examples of this expanding field of research are presented here:

### Recombinant vWF

Following the cloning of vWF, its complex protein structure, post-translational modifications and multimerization have been characterized in CHO cells (93). In this expression system, recombinant vWF (rvWF) undergoes glycosylation and dimerization through C-terminal disulfide bond formation in the endoplasmic reticulum. Within the Golgi, post-translational modifications are completed including multimerization and removal of the propeptide. High expression of rvWF in CHO cells demonstrated that it formed heteromultimers, mixtures of mature vWF and pro-vWF due to incomplete propeptide removal under such conditions (94). Co-expression of furin in rvWF-expressing CHO cells improved the processing; however, at very high expression rates additional downstream processing of the purified rvWF is required. Plasma vWF is known to undergo proteolytic clipping between Tyr842–Met843 through the action of ADAMTS13. In contrast, rvWF exhibits no evidence of such proteolysis. Thus its multimeric pattern is more typical of platelet/endothelial-stored vWF. In vitro studies have demonstrated that rvWF has similar functional activities to pdvWF. Biological haemostatic activity has been demonstrated in several animal models including murine, canine and porcine vWF-deficient models (94).

Whereas, the clinical development of rvWF would provide von Willebrand disease patients the same recombinant option afforded those with haemophilia, it also holds promise for advanced rFVIII products. There is considerable interest in extending the half-life of FVIII, thereby improving efficacy and adherence with prophylactic regimens for haemophilia A (95). Due to inherent problems with direct modification of FVIII, an indirect approach has been investigated in which rvWF bioengineered for an extended half-life could be infused along with rFVIII, in turn extending the half-life of rFVIII in plasma (96). Due to the substantially larger market potential for such an application, this may be the motivation necessary to see rvWF through to commercialization.

### Recombinant factor XIII (rFXIII)

FXIII is a transglutaminase circulating in plasma as a heterotetramer of two A and two B subunits noncovalently associated and bound to fibrinogen (97). It promotes clot stability primarily through crosslinking of fibrin monomers. The FXIII B subunit is found in excess in plasma and is believed to stabilize the structure and prevent proteolysis of the A subunit. Congenital FXIII deficiency is a very rare autosomal recessive disorder, affecting only one in 1–5 million worldwide. The majority of the reported mutations are found in the A subunit. It causes severe bleeding manifestations lifelong including a high rate (20–40%) of spontaneous intracranial haemorrhage. However, because of the long half-life of FXIII (8–12 days), it has proven to be amenable to long-term prophylaxis with cryoprecipitate, fresh frozen plasma as well as plasma-derived FXIII concentrates. Plasma-derived FXIII concentrates have been prepared from placenta (A subunit homodimers) and plasma (A and B subunit heterotetramers).

The primary structure of the A and B subunits was deter-

mined from a combination of cDNA and amino acid sequence analysis and the individual genes eventually cloned (98). Human rFXIII A subunit homodimer (rFXIII) was expressed efficiently in yeast. The rFXIII associates with plasma FXIII B subunits to form the stable heterotetramer. Safety, pharmacokinetics and immunogenicity have been studied in healthy volunteers (99). A phase I clinical trial in adults with congenital FXIII deficiency showed a half-life similar to plasma derived FXIII (8.5 days), normalized the urea clot lysis assay, and improved clot strength and resistance to fibrinolysis as determined by thromboelastography (97). No serious adverse events were observed, including no bleeding or thrombotic events and no antibodies to FXIII or yeast proteins. However, toxicity studies in cynomolgus monkeys demonstrated a generalized coagulopathy and death in monkeys treated with supraphysiological doses. This has been associated with the formation of high-molecular-weight protein complexes consisting of plasma proteins crosslinked by FXIII providing a potential biomarker for toxicity in future clinical trials (100).

### Bioengineered recombinant clotting factors

At the very heart of recombinant DNA technology is not merely the goal to mimic native proteins but to exploit insights into their structure and function and engineer targeted modifications to enhance their function. Whereas rBDD-FVIII was the first foray into this technology, multiple approaches are now being explored for improvements to rFVIII, rFIX and rFVIIa. These have been extensively reviewed elsewhere (95, 101–103).

### Gene therapy

The past decade has seen the first wave of clinical trials applied to subjects with haemophilia. The first attempt for haemophilia B was performed at the Fudan University in China in 1991 (104). Subsequently, five phase I clinical trials have been conducted (see review [105]). The strategies have included in-vivo delivery of genes to express FVIII and FIX utilizing viral vectors and an ex-vivo approach employing plasmid transfection of human cells that were then transplanted back into the recipients. Recombinant technology was foundational to realizing these trials and in one case included the use of bioengineered FVIII (106). Although the trials demonstrated the potential safety of such ap-

proaches, stable expression of therapeutic levels of FVIII or FIX have not yet been achieved. Ongoing research will lead to improved vector systems, an improved understanding of the immunology of gene transfer technology and possibly include the application of new bioengineered rFVIII or rFIX (107).

### Alternative expression systems and transgenic livestock

The lack of availability and affordability of clotting factor concentrates remains a major disappointment of the recombinant era in haemophilia. However, there remains enthusiasm to explore alternative expression systems including enhanced cell culture systems and transgenic animal technology to address this issue. In one example, CHO recombinant protein expression has been enhanced by flanking the gene of interest with transcriptional control regions from a highly expressed gene in CHO cells, Chinese hamster elongation factor-1 $\alpha$  (CHEF-1) (108). This strategy has led to at least a 10-fold increase in the expression of several secreted proteins and is currently being exploited to increase the efficiency of rFIX expression for commercial production.

Transgenic animals offer the potential to replace commercial bioreactors for recombinant protein production (109). The typical cell density of genetically engineered cells achieved within a bioreactor is relatively low at approximately  $5 \times 10^6 \text{ ml}^{-1}$ . In contrast, animal mammary tissues are capable of producing high concentrations of secreted proteins with cell densities in the  $10^9 \text{ ml}^{-1}$  range, 2–3 orders of magnitude greater than can be achieved with cell culture-based commercial recombinant production systems. This has been exploited for a number of recombinant proteins including fibrinogen and rFIX. This technology has also been partnered with novel bioengineered rFVIII molecules with enhanced secretion efficiency to further improve expression (110). This technology has been promoted by the US National Haemophilia Foundation's Medical and Scientific Advisory Committee (111). Recently, the world's first transgenically produced recombinant protein, a recombinant antithrombin III from the milk of transgenic goats, was approved by both the European and USA federal regulators (112). This may open the door for the future approval of haemophilia therapies derived from such technology yielding low cost recombinant clotting factors that can be utilized in the developing world.

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