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ORIGINAL ARTICLE

Pharmacokinetics, immunogenicity, safety, and preliminary efficacy of subcutaneous turoctocog alfa pegol in previously treated patients with severe hemophilia A (alleviate 1)

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Abstract

Background: The current standard of care for patients with hemophilia A is regular prophylaxis with factor VIII (FVIII) administered intravenously. Interest in subcutaneous (s.c.) administration, to potentially increase convenience, reduce the treatment burden and improve compliance, is increasing.

Objectives: Evaluate the pharmacokinetics (PK), immunogenicity, safety, and preliminary efficacy of s.c. administration of turoctocog alfa pegol (s.c. N8-GP) in adult or adolescent previously treated patients (PTPs) with severe hemophilia A (alleviate 1; NCT02994407).

Patients/Methods: In part A, 24 PTPs received a single dose of s.c. N8-GP (12.5, 25, 50, or 100 IU/kg) with 6 patients per cohort. PK modelling of data from part A supported a suitable dose for part B. Part B comprised a multiple dose trial in 26 PTPs; patients <60 kg received 2000 IU and patients ≥60 kg received 4000 IU s.c. N8-GP daily for 3 months.

Results: Single-dose s.c. N8-GP supported dose linearity. Daily prophylaxis with s.c. N8-GP appeared well tolerated and efficacious, achieving a mean trough FVIII activity close to 10% at steady state. Five patients developed anti-N8-GP binding antibodies after 42 to 91 exposure days, one of whom developed an inhibitor to FVIII. Anti-N8-GP antibody appearance was associated with a decline in FVIII plasma activity in four of the five patients. Five patients reported a total of nine treatment-requiring bleeding episodes during prophylaxis.

Conclusions: Subcutaneous administration of N8-GP is associated with a high incidence of antibodies in PTPs with severe hemophilia A. Further clinical development of s.c. N8-GP has been suspended.

KEYWORDS

antibodies, factor VIII, hemophilia A, subcutaneous infusions

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1 | INTRODUCTION

Coagulation factor VIII (FVIII) replacement therapy—particularly the introduction of recombinant FVIII (rFVIII) products in the 1990s—transformed the landscape of hemophilia A care.[1] The availability of prophylactic FVIII replacement therapy has reduced the incidence of hemophilic arthropathy and overall morbidity and turned a life-threatening disease into a chronic yet manageable condition.[2-5] The life expectancy of people with hemophilia (PWH) is now approaching that of the general population,[6] and new treatments are increasingly focused on reducing the burden of care.[7] The introduction of extended half-life (EHL) rFVIII therapies has increased bleed protection and FVIII trough levels, with potential for improved quality of life.[7] Furthermore, EHL rFVIII therapies have improved convenience of prophylaxis because of the less-frequent dosing regimens required, compared with standard half-life products. However, the relative increase in half-life of currently available EHL FVIII products for hemophilia A treatment is only modest, compared with that of EHL factor IX (FIX) products used to treat hemophilia B.[7] All EHL products are currently administered intravenously.[7]

Regular intravenous (i.v.) administration of rFVIII is challenging, particularly for young children and the elderly, due to restricted vascular access.[1,7] An alternative to i.v. is subcutaneous (s.c.) administration, which has the potential to further improve the convenience of prophylaxis by reducing the need for i.v. access and central venous access devices (CVADs).[8] Furthermore, s.c. administration is less invasive and may be less painful than i.v. treatment due to the shorter and thinner injection needles used.[8,9] Easier administration of rFVIII has the potential to increase adherence and, therefore, improve patient outcomes.[10]

A major limitation of s.c. administration of unmodified FVIII is that its bioavailability is low in preclinical models, due to insufficient absorption and rapid degradation.[11-13] Consequently, its clinical application has not been successful.[9] Alternative subcutaneously administered hemophilia treatment options include bispecific antibodies that bridge activated FIX and factor X (eg, emicizumab)[14] or agents that inhibit endogenous anticoagulant pathways (eg, fitusiran and concizumab).[15] However, the safety and efficacy of these treatments outside of the clinical trial setting has not been established.[15]

N8-GP (turoctocog alfa pegol; Novo Nordisk, Bagsværd, Denmark) is an EHL rFVIII approved in the United States for prophylaxis and treatment of bleeding episodes in patients with hemophilia A.[16,17] In adults with severe hemophilia A, the plasma half-life of N8-GP following i.v. administration is approximately 19 hours, which equates to a 1.6-fold prolonged half-life compared with standard half-life rFVIII.[18] N8-GP is manufactured by glycoPEGylation of turoctocog alfa, whereby the terminal sialic acid on an O-glycan in the truncated B-domain is replaced by a conjugated sialic acid containing a 40-kDa polyethylene glycol (PEG) moiety.[16] Clinical trials have shown that i.v. N8-GP is efficacious and well tolerated in previously treated patients (PTPs), with a low incidence of inhibitors reported.[19-21]

Essentials

- Subcutaneous (s.c.) factor VIII (FVIII) administration may reduce the treatment burden of hemophilia A.
- Alleviate 1 investigated the s.c. administration of turoctocog alfa pegol (N8-GP) in adults.
- Steady-state FVIII activity levels of ~10% suggest a potential for efficacious s.c. FVIII prophylaxis.
- Five out of 26 patients developed anti-N8-GP binding antibodies, one of whom developed inhibitors to FVIII.

The two major challenges of s.c. administration of large-protein pharmaceuticals are immunogenicity and bioavailability of the drug to achieve therapeutic levels.[22] The development of high-affinity immunoglobulin G (IgG) anti-FVIII antibodies to exogenous FVIII remains the most serious complication of hemophilia A management.[23] Inhibitors neutralize FVIII, resulting in ineffective FVIII replacement therapy and poor bleed control.[23,24] Preclinical studies suggest that coagulation factor proteins administered subcutaneously are, potentially, more immunogenic than those administered intravenously;[25] however, the immunogenicity of FVIII in humans following s.c. administration is unknown.

Preclinical studies have shown s.c. N8-GP, in contrast to s.c. standard half-life FVIII, to have a bioavailability of 24% and 26% and a half-life of 14 and 15 hours in hemophilia A mice and cynomolgus monkeys, respectively.[9] Compared with standard half-life rFVIII, glycoPEGylation appears to increase the bioavailability of s.c. N8-GP and also may be responsible for increasing the solubility of N8-GP under isotonic conditions.[9] Based on preclinical pharmacokinetic (PK) data, s.c. N8-GP daily was predicted to provide FVIII trough levels of 2.5-10% in adults, which would convert severe hemophilia patients to a moderate or potentially even mild phenotype.[9]

Here, we present results from the alleviate 1 trial, the first-in-human clinical trial to investigate s.c. N8-GP for prophylactic use in patients with severe hemophilia A. Its aim was to evaluate the PK, immunogenicity, safety, and preliminary efficacy following single and multiple doses of s.c. N8-GP in PTPs.

2 | MATERIALS AND METHODS

2.1 | Trial design and treatments

Alleviate 1 was a phase 1, multicenter, PK, safety and preliminary efficacy trial in PTPs with severe hemophilia A conducted in 25 sites in 9 countries (Austria, Bulgaria, France, Germany, Japan, Serbia, Turkey, United Kingdom, and United States). The primary objective was to assess the safety of s.c. N8-GP, including immunogenicity. The trial was approved by all relevant independent ethics committees and institutional review boards. Written informed consent was obtained from all participants or their legally authorized

representatives before any trial-related activities commenced. The trial was conducted in accordance with the declaration of Helsinki[26] and Good Clinical Practice[27], and is registered at www.clinicaltrials.gov (alleviate 1 NCT02994407).

The trial consisted of two parts. Part A was a single-dose dose-escalation trial and part B was a multiple-dose trial (Figure 1). Participants in part A received a single dose of s.c. N8-GP 12.5, 25, 50, or 100 IU/kg. For assessment of local tolerability, part A was double-blinded with regard to receipt of active treatment (N8-GP) or placebo. Patients received s.c. N8-GP and placebo immediately after one another in a blinded manner, above and below the umbilicus.

Time was allowed between part A and B to permit a global amendment to the dosing regimen. Patients in part B were treated with a daily fixed dose of s.c. N8-GP for 13 weeks and the final follow-up visit was scheduled 28 days after the last dosing visit (Figure 1). Population PK modelling was used to determine doses for part B that predicted 95% of patients would have a FVIII trough level of ≥3%. Patients <60 kg received 2000 IU (one injection) and patients ≥60 kg received 4000 IU (two injections) of s.c. N8-GP daily. Patients in part A were allowed to enroll into part B. Dose adjustments in part B were permitted at the discretion of the investigator, based on FVIII trough levels (target trough level >3%) or bleeding criteria (≥1 spontaneous treatment-requiring bleed between Visit 4 and 8) and could take place any time between Visit 4 and 8, 2-13 weeks after the first dose of s.c. N8-GP. Dosing could be increased up to 6000 IU, but could not exceed 100 IU/kg/day.

2.2 | Key eligibility criteria

Males, age ≥18 years (part A) and ≥12 years (part B) with severe hemophilia A (FVIII activity <1%) and without inhibitors to FVIII (<0.6 Bethesda units [BU]) were enrolled. Part B involved a staggered

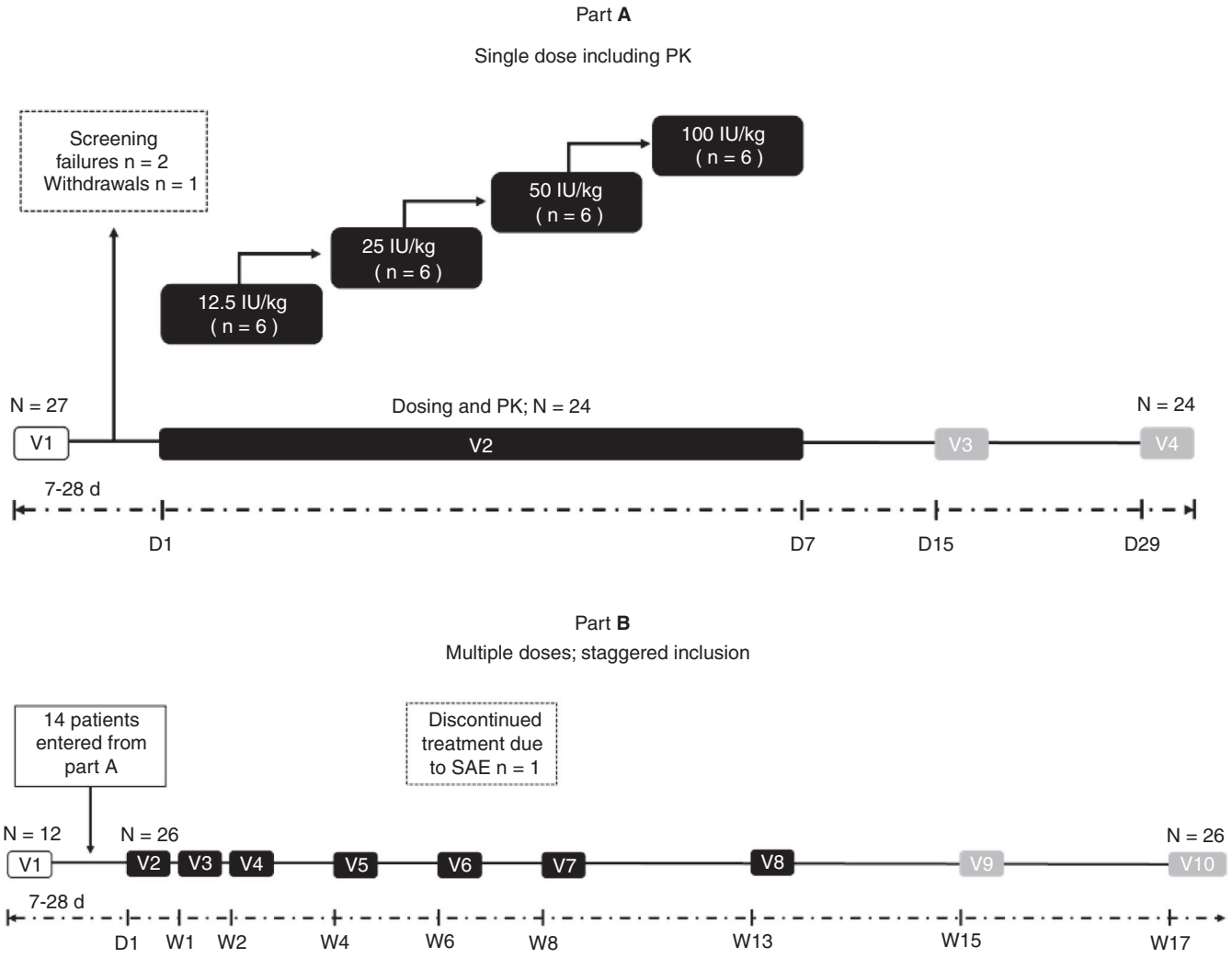

FIGURE 1 Alleviate 1 trial design and patient flow. In part A, 27 patients were screened and 24 started treatment. In part B, 26 patients were included and started on trial product. Of these, a total of 14 patients continued from part A to part B and 12 additional patients were included. PK assessments were performed during Visits 2-4 in part A and at Visits 2-10 in part B. Clear boxes indicate screening visits, black boxes indicate dosing visits, and gray boxes indicate follow-up visits. D, day; PK, pharmacokinetics; SAE, serious adverse event; V, visit; W, week

enrollment, as the first six patients had to be ≥18 years old. All participants were PTPs with >150 exposure days (EDs) to any FVIII product. Patients with a history of inhibitors or immune deficient patients due to HIV (defined as a CD4+ lymphocyte count ≤200/μL or viral load ≥400 000 copies/mL) were not eligible for the trial.

2.3 | Objectives, endpoints and assessments

PK parameters evaluated in parts A and B included area under the activity-time curve (AUC), maximum FVIII activity of s.c. N8-GP (C_{max}), average FVIII activity of s.c. N8-GP at steady state (C_{avg}), and half-life of s.c. N8-GP ($t_{½}$). FVIII activity was measured using a chromogenic assay with a product-specific standard. The PK evaluation of s.c. N8-GP parameters included 12 sampling points in part A, with blood drawn prior to dosing and then at 1, 2, 3, 5, 8, 24, 48, 72, 96, 120, and 144 hours postdose. In part B, nine sampling points were scheduled for all patients, beginning prior to dosing and then at 1, 4, 8, 12, 24, 48, 72, and 96 hours postdose. The primary objective of alleviate 1 was to evaluate the safety of s.c. N8-GP, and the primary endpoint was the frequency of adverse events (AEs) reported after first exposure to s.c. N8-GP until 28 days after last exposure, evaluated in parts A and B. Safety was assessed via reporting of AEs, vital signs, electrocardiogram, physical examination, and safety laboratory parameters. AEs were categorized according to severity, location, causality, and final outcome. The number of treatment-emergent AEs (TEAEs) was also reported. AEs were considered treatment-emergent if they occurred on or after the first day of trial product administration, and until 7 days after the last administration of trial product.

Immunogenicity assessment included testing for FVIII inhibitors, anti-N8-GP binding antibodies, anti-N8-GP antibody-specific isotype, anti-PEG antibodies, and anti-host cell protein (HCP) antibodies. The incidence of FVIII inhibitors and anti-N8-GP antibodies was evaluated in parts A and B. Blood samples for detection of inhibitors were drawn at each visit prior to dosing and analyzed at a central laboratory (Laboratorium für Klinische Forschung GmbH, Germany) using the Nijmegen-modified Bethesda assay, including heat treatment of controls and samples.[28,29] In accordance with the European Medicines Agency guideline,[30] a patient was considered to have FVIII inhibitors if they had tested positive for inhibitors (≥0.6 BU) in two consecutive test samples analyzed at the central laboratory. These patients were withdrawn from s.c. N8-GP treatment but remained in the trial following the planned visit schedule. The presence of anti-N8-GP antibodies was determined by a validated radioimmunoassay, and presence of antibodies confirmed in a confirmatory assay. In the confirmatory assay, specificity of the antibodies was confirmed by adding an unlabeled drug to the assay. If the antibodies were specific, the unlabeled drug would compete with the radioactively labeled drug, resulting in a reduction of the signal. Positive antibody samples were further characterized, eg, for immunoglobulin isotype by a direct enzyme-linked immunosorbent assay (ELISA). A direct ELISA was also used to measure anti-PEG antibodies. All antibody testing assays were developed by Novo Nordisk and conducted in-house or at contract research organizations. Other safety assessments included physical examinations and clinical laboratory tests (hematology, biochemistry, coagulation parameters, and urinalysis).

Efficacy endpoints were evaluated in part B only. The number of treatment-requiring bleeding episodes (bleeding episodes treated with i.v. rFVIII) during part B was summarized together with the duration of exposure. The details of the bleeds, including the location, type, severity, time of onset, duration, time of bleed since the prophylaxis dose, as well as the time from bleed onset to first treatment dose were recorded in either the medical records or in the patient's diary. The consumption of s.c. N8-GP for prophylaxis treatment was also recorded.

2.4 | Statistical methods

No formal sample size calculation was performed and data analyses were based on descriptive statistics. PK endpoints were based on the full analysis set, comprising all patients with a minimum of one evaluable PK profile. Evaluation of dose linearity for s.c. N8-GP was based on AUC and C_{max} data derived from part A, and was conducted using an analysis of variance (ANOVA) on log-transformed parameter values with log dose as a covariate. Slope estimates were calculated with 95% confidence intervals (CIs). AUC and C_{max} data derived from part B were analyzed using a mixed effects model on the log-transformed values, with visit and dose as fixed effects and patient as random effect. Estimated means were calculated with 95% CIs for each visit.

The terminal elimination rate constant (λz) was estimated using a population-based approach where data below the lower limit of quantification (LLoQ) were treated as left-censored data. This allowed the estimation of the terminal rate constant for patients with too few data above the LLoQ, which would not have been possible based on single-patient data. This estimation was performed separately for part A and part B. Safety endpoints were based on the safety analysis set, which included all patients exposed to s.c. N8-GP. Serious AEs (SAEs), causality, and severity were summarized for part A and part B separately.

3 | RESULTS

3.1 | Subject disposition and baseline characteristics

Baseline characteristics for patients in parts A and B are shown in Table 1. In part A, 24 patients received a single dose of s.c. N8-GP: either 12.5, 25, 50, or 100 IU/kg (n = 6 per cohort). Most patients received multiple injections to achieve the targeted dose and placebo. All patients who were dosed completed the trial (Figure 1). The mean age of patients was 36.8 years and 83.3% were white. A total of 26 patients were enrolled in part B, 14 of whom had completed part A. The mean age of patients in part B was 33.9 years and 80.8% were white. One patient in part B received 2000 IU (body weight <60 kg) and 25 patients received 4000 IU (body weight ≥60 kg) for a total

TABLE 1 Patient demographics

Part A				
	12.5 IU/kg	**25 IU/kg**	**50 IU/kg**	**100 IU/kg**
Number of patients, N	6	6	6	6
Age in years, mean (SD)[a]	36.0 (10.5)	37.8 (15.8)	34.7 (16.7)	38.7 (16.1)
Race, N (%)				
White	6 (100.0)	4 (66.7)	5 (83.3)	5 (83.3)
Asian	-	2 (33.3)	-	-
Black or African American	-	-	-	1 (16.7)
Not known[b]	-	-	1 (16.7)	-
Weight in kg, mean (SD)	93.7 (5.1)	83.2 (11.2)	79.6 (17.3)	80.4 (7.4)
BMI in kg/m^2, mean (SD)	26.2 (2.3)	29.8 (5.0)	25.4 (5.0)	26.5 (3.3)
FVIII genotype, n (%)				
Small deletion	1 (16.7)	1 (16.7)	1 (16.7)	-
Small duplication	-	-	1 (16.7)	-
Intron 1 inversion	-	1 (16.7)	-	-
Intron 22 inversion	2 (33.3)	3 (50.0)	3 (50.0)	4 (66.7)
Missense mutation	2 (33.3)	1 (16.7)	-	1 (16.7)
Nonsense mutation	-	-	-	1 (16.7)

Part B	
	Total
Number of patients, N[c]	26
Age in years, mean (SD)	33.9 (15.4)
Race, N (%)	
White	21 (80.8)
Asian	2 (7.7)
Black or African American	1 (3.8)
Other	1 (3.8)
Not known[b]	1 (3.8)
Weight in kg, mean (SD)	78.9 (13.6)
BMI in kg/m^2, mean (SD)	26.0 (4.2)
FVIII genotype, n (%)	
Small duplication	2 (7.7)
Intron 1 inversion	1 (3.8)
Intron 22 inversion	14 (53.8)
Missense mutation	4 (15.4)
Nonsense mutation	2 (7.7)

Abbreviations: BMI, body mass index; FVIII, factor VIII; SD, standard deviation.

[a]All patients, age ≥18 years.

[b]Information not disclosed by the patient.

[c]Included n = 3 adolescent patients and n = 23 patients age ≥18 years.

of 3 months. One patient had a dose adjustment from 4000 IU to 6000 IU (not based on the dose-adjustment criteria). All patients completed part B with a total of 90.35 mean EDs to s.c. N8-GP. The trial began on 30 January 2017 and the last patient last visit was on 15 October 2018.

3.2 | PK (parts A and B)

PK parameters are shown in Table 2. In part A, the geometric mean half-life of N8-GP after s.c. administration was 20.6 and 21.6 hours following a dose of 50 and 100 IU/kg, respectively. Slope estimates

TABLE 2 PK parameters in parts A and B

Part A				
PK parameter	**12.5 IU/kg**	**25 IU/kg**	**50 IU/kg**	**100 IU/kg**
$AUC_{0\text{-}Inf}$ (IU*h/dL)				
Estimate (95% CI)	147.1 (88.6; 244.0)	149.1 (89.8; 247.3)	225.1 (135.7; 373.5)	837.1 (504.5; 1389.0)
Geometric mean (CV%)	147.1 (94.8)	149.1 (49.3)	225.1 (39.4)	837.1 (71.4)
C_{max} (IU/dL)				
Estimate (95% CI)	1.3 (0.8; 2.2)	2.5 (1.4; 4.3)	4.6 (2.7; 7.9)	15.2 (8.8; 26.3)
Geometric mean (CV%)	1.3 (106.9)	2.5 (62.8)	4.6 (32.6)	15.2 (75.6)
$t_{½}$ (h)				
Estimate (95% CI)	74.0 (62.2; 88.1)	29.3 (24.6; 34.8)	20.6 (17.3; 24.5)	21.6 (18.2; 25.8)
Geometric mean (CV%)	74.0 (25.5)	29.3 (27.4)	20.6 (13.3)	21.6 (12.0)
Part B				
PK parameters	**Dosing visit (Visit 2)**	**Week 1 (Visit 3)**	**Week 13 (Visit 8)**	
$AUC_{0\text{-}24h}$ (IU*h/dL)				
Estimate (95% CI)	97.3 (73.1; 129.6)	284.6 (212.9; 380.5)	228.5 (170.1; 307.0)	
Geometric mean (CV%)	—	—	235.9 (86.6)	
C_{max} (IU/dL)				
Estimate (95% CI)	5.7 (4.3; 7.5)	13.6 (10.3; 18.0)	11.5 (8.6; 15.3)	
Geometric mean (CV%)	5.7 (90.0)	13.9 (64.4)	11.9 (80.2)	
C_{avg} (IU/dL)				
Estimate (95% CI)	—	11.9 (9.0; 15.6)	9.6 (7.3; 12.7)	
Geometric mean (CV%)	—	12.2 (62.7)	9.8 (85.5)	

FVIII activity was measured using the chromogenic assay with a product-specific standard. Overall estimate and 95% CI data were derived from an ANOVA model with log (PK parameter) as response and log (dose) as covariate.

Abbreviations: AUC, area under the activity-time curve; ANOVA, analysis of variance; C_{avg}, average FVIII activity at steady state; CI, confidence interval; C_{max}, maximum FVIII activity; CV, coefficient of variation; PK, pharmacokinetics; $t_{½}$, half-life.

and 95% CIs from an ANOVA model on log-transformed $AUC_{(0\text{-}inf)}$ and C_{max} from part A (0.8 [0.4; 1.2] IU*h/dL and 1.2 [0.8; 1.5] IU/dL, respectively), with log dose as covariate, supported a linear dose response for s.c. N8-GP (Figure 2A). Geometric mean estimated C_{max} values increased from 1.3 IU/dL following 12.5 IU/kg s.c. N8-GP to 15.2 IU/dL after 100 IU/kg s.c. N8-GP (Table 2). The pattern was consistent with dose linearity.

In part B, steady-state FVIII activity was measured at Visit 3, 7 days after the initial dose of s.c. N8-GP, and again at Visit 8, after 3 months of treatment. Steady state refers to the situation where the overall absorption of a drug is in dynamic equilibrium with its elimination.[31] Steady state after multiple dosing is reached after four to five half-lives, for a drug following linear kinetics.[32,33] At Visit 3, the estimated (95% CI) mean FVIII activity (C_{avg}) was 11.9 (9.0; 15.6) IU/dL. Mean FVIII activity declined to 9.6 (7.3; 12.7) IU/dL at the final dosing visit (Visit 8) (Table 2) due to the development of anti-N8-GP antibodies in five patients (see Table 4). Overall, treatment with s.c. N8-GP in part B resulted in a mean pre-dose (trough) FVIII activity close to 10% throughout the 13-week study period (Figure 2B).

3.3 | Immunogenicity and safety

A total of 16 AEs were reported in 11 patients in part A, and 45 AEs were reported in 17 patients in part B, including 15 TEAEs in part A and 40 TEAEs in part B. Of these, 11 (part A) and 7 (part B) were considered treatment-related (Table 3). The most common TEAEs in part A that were possibly or probably treatment related were injection-site reactions (bruising, erythema, and pain; 6 events in 5 patients), all of which resolved. A total of 11 injection-site reactions were recorded in part A, 8 of which were from placebo. FVIII inhibition, oral paresthesia, feeling cold, injection-site bruising, increased alanine aminotransferase, back pain, and headache (n = 1 for each) were each considered possibly or probably treatment related in part B; all treatment-related AEs resolved except for the FVIII inhibitor. The inhibitor patient is described below.

Two SAEs were reported in two patients (one patient developed a FVIII inhibitor and one patient had severe arthralgia [not considered related to s.c. N8-GP]), both during part B. The patient with severe arthralgia was hospitalized with left knee pain; however, an underlying explanation for the joint pain could not be established and the patient

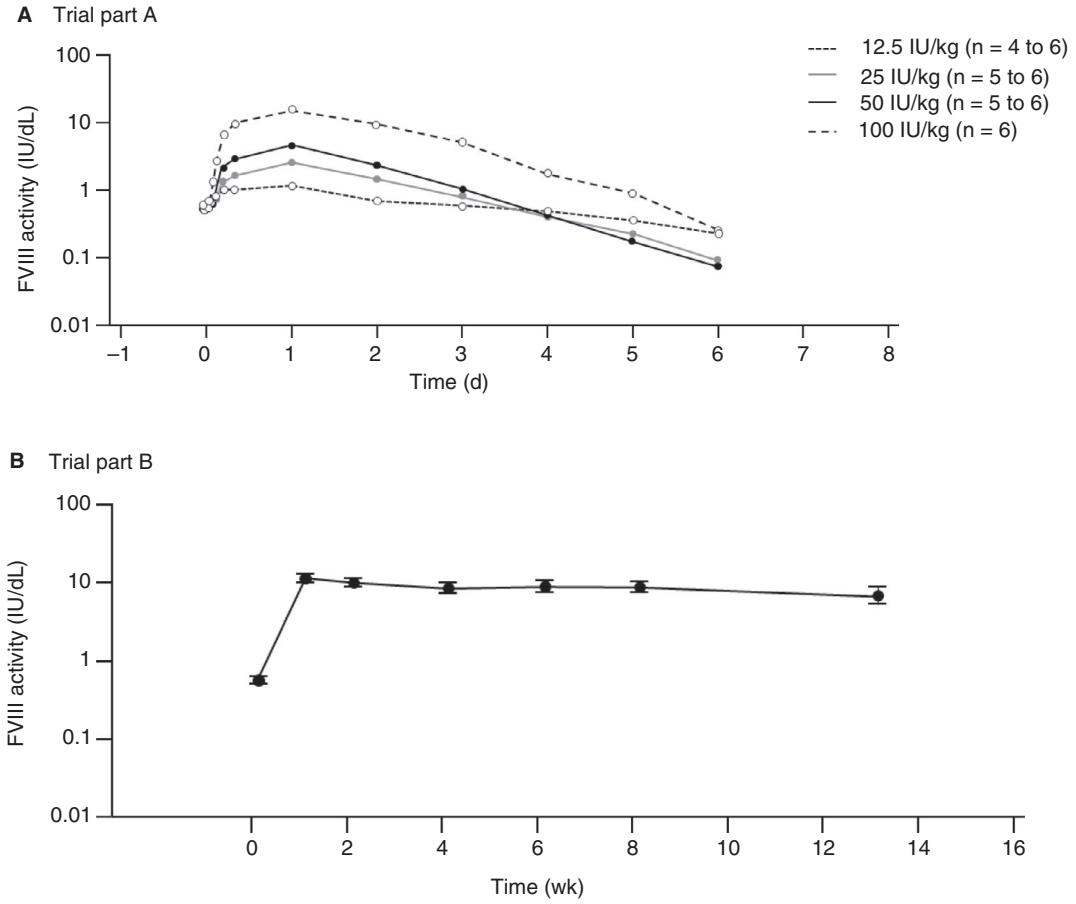

FIGURE 2 PK assays were performed using a chromogenic assay with a product-specific standard. Data represent geometric mean (CV%) FVIII activity and are plotted on a log scale. Part A: mean pharmacokinetic profiles of FVIII activity after four different single doses of s.c. N8-GP. Values below LLOQ (0.9 IU/dL) were predicted using population-based approach. Part B: mean pre-dose FVIII activity at Visit 2 (first dose in part B), Visit 3 (after 7 days of daily doses), Visit 4, Visit 5, Visit 6, Visit 7, and Visit 8. Patients received a fixed dose of s.c. N8-GP at 4000 IU/day (except for one patient who received 2000 IU/day). Error bars are standard error of the mean. Geometric mean (CV%) = 11.5 (64.6) IU/dL. min; max = 3.4; 45.4. CV, coefficient of variation; FVIII, factor VIII; LLOQ, lower limit of quantification; PK, pharmacokinetics; s.c. subcutaneous

was discharged. This SAE had resolved by the end of the trial. No AEs were fatal and none led to patient withdrawal from the trial. No safety concerns with regard to vital signs, hematology, biochemistry, coagulation parameters, or urinalysis were reported in part A or B.

No patients developed FVIII inhibitors in part A following administration of s.c. N8-GP. In part B, a 36-year-old patient with an underlying hemophilia A missense mutation developed a low-titer FVIII inhibitor, detected after 49 EDs (at an unscheduled visit between Visit 6 and 7), which reached a maximum inhibitor titer of 2.6 BU during the trial (Figure 3), at which point in time the patient had no measurable FVIII activity. This patient discontinued treatment with s.c. N8-GP when the inhibitor was identified and was treated instead with bypassing agents. The patient completed the trial and entered the immune tolerance induction trial after the end-of-treatment visit, in which he was treated with turoctocog alfa. In total, five patients, including the patient who developed a FVIII inhibitor, tested positive for anti-N8-GP binding antibodies in part B, detected after 42, 89 (n = 2), 56 and 91 EDs (Table 4 and Figure 3). Isotype development was evaluated for these patients and N8-GP–specific IgG4 was detected in four out of the five patients who had binding antibodies, including the inhibitor patient. At all visits, FVIII trough levels were measured and the presence of high levels of antibodies and N8-GP-specific IgG4 correlated with a decrease in FVIII activity (Table 4 and Figure 4).

Two patients in part A had pre-existing anti-PEG binding antibodies, one of whom was only positive for antibodies prior to administration of s.c. N8-GP. The other patient remained positive for anti-PEG antibodies throughout the trial. Four patients tested positive for anti-PEG antibodies in part B after 7, 14, 28, and 91 EDs. Of these, two patients still had anti-PEG antibodies detected at the end-of-treatment visit, while the other two cases were transient. One patient with anti-PEG antibodies had inhibitors to FVIII. Two patients tested positive for anti-HCP antibodies in part A, one prior to and one after dosing with s.c. N8-GP.

3.4 | Efficacy (part B only)

Mean total monthly consumption of s.c. N8-GP was 1477.3 IU/kg per patient (standard deviation: 255.5 IU/kg). During s.c. N8-GP

TABLE 3 Overview of adverse events in part A and B

	Part A (n = 24)	Part B (n = 26)
AEs, n (%) e	11 (45.8) 16	17 (65.4) 45
Severe	0 (0) 0	1 (3.8) 1
Moderate	0 (0) 0	9 (34.6) 12
Mild	11 (45.8) 16	14 (53.8) 32
SAEs, n (%) e	0 (0) 0	2 (7.7) 2
Treatment-related AEs, n (%) e	7 (29.2) 11	7 (26.9) 7
TEAEs, n (%) e	10 (41.7) 15	17 (65.4) 40
Severe	0 (0) 0	1 (3.8) 1
Moderate	0 (0) 0	9 (34.6) 12
Mild	10 (41.7) 15	14 (53.8) 27

Abbreviations: %, percentage of patients with adverse event; AE, adverse event; e, number of adverse events; n, number of patients with adverse event; SAE, serious adverse event; TEAE, treatment-emergent adverse event.

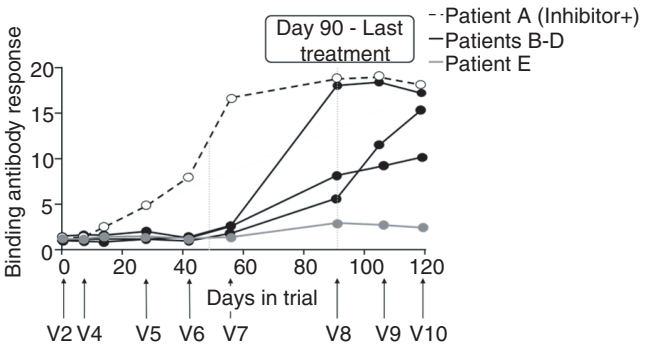

FIGURE 3 Binding antibody response of patients who developed anti-N8-GP antibodies in part B. Binding antibody response was calculated as percentage bound antibody over total. Response was measured at each clinic visit. Anti-N8-GP antibodies were detected between 42 and 91 s.c. N8-GP exposure days. The dashed line represents patient A, who developed FVIII inhibitors, had high levels of binding antibodies, N8-GP-specific IgG4, and affected FVIII trough levels. The black lines represent patients B-D, who had high levels of binding antibodies and also had N8-GP-specific IgG4 detected, as well as affected FVIII trough levels. The gray line represents patient E who had low levels of binding antibodies detected, with no IgG4 detected and no effect on FVIII activity levels. FVIII, factor VIII; V, visit

prophylaxis, 80% of patients reported zero bleeds. Five patients reported a total of nine treatment-requiring bleeding episodes during prophylaxis treatment with s.c. N8-GP. Of these, four treatment-requiring bleeding episodes occurred in two patients with anti-N8-GP antibodies. Six of the bleeds were spontaneous and all bleeds were mild or moderate in severity. Seven of the bleeds were in joints. A total of seven treatment-requiring bleeds occurred in three of the five patients with anti-N8-GP antibodies during the trial, including the safety follow-up.

4 | DISCUSSION

Alleviate 1 was a first-in-human trial that evaluated the s.c. administration of a PEGylated EHL FVIII replacement therapy for the management of patients with severe hemophilia A. Subcutaneous N8-GP was intended as a new prophylaxis treatment option for PWH, providing improved FVIII coverage and long-term outcomes with a simple and convenient method of administration. Data from alleviate 1 show that a single dose of s.c. N8-GP supported dose linearity, and daily prophylaxis with s.c. N8-GP appeared well tolerated and efficacious; however, anti-N8-GP antibodies were detected in five patients, one of whom had detectable FVIII inhibitors.

Data from part A were consistent with dose linearity following single-dose administration of s.c. N8-GP, and steady state was achieved in part B at Visit 3, 1 week after the first dose. Steady state after multiple dosing is reached after 4 to 5 half-lives for a drug following linear kinetics[32,33] and refers to the situation in which the overall absorption of a drug is in dynamic equilibrium with its elimination.[31] The half-life of s.c. N8-GP in humans was similar to that of i.v. N8-GP - 21.6 versus approximately 19 hours, respectively.[20] As predicted from preclinical models,[9] daily treatment with s.c. N8-GP shifted most patients from a severe to mild hemophilia A phenotype, with mean FVIII activity >10% at all times and little fluctuation between trough and peak activity (Figures 2 and 4). Converting the phenotype of severe hemophilia patients is a key goal of prophylaxis to improve patient outcomes over the long term.[34]

The development of inhibitors remains the most significant complication of FVIII replacement therapy.[23] Anti-N8-GP antibodies were detected in five patients after 42-91 EDs in part B, one of whom had detectable FVIII inhibitors 49 EDs after the first dose of s.c. N8-GP. The inhibitor patient discontinued treatment and has since entered an immune tolerance induction trial for treatment with turoctocog alfa. The four patients with high levels of anti-N8-GP binding antibodies also experienced a decrease in FVIII activity. A meta-analysis of 41 studies concluded that the rate for clinically relevant *de novo* inhibitor development in PTPs treated with i.v. FVIII is as low as 2.06 per 1000 patient-years.[35]

"Late" immunogenic responses have been reported previously in PTPs introduced to two i.v. FVIII products processed with a new manufacturing method.[36-38] After the introduction of this new pasteurized FVIII concentrate (FVIII CPS-P) in the Netherlands in 1990, an increase in the occurrence of inhibitors in previously treated hemophilia A patients was reported after 50-1000 EDs.[36,37] Unlike the typical immunological response, these antibody titers showed a rapid decline following a switch to a different FVIII product.[37] The same was true following the introduction of FVIII-SDP (Octavi SDPlus, Octapharma, Lachen, Switzerland) in Belgium and Germany in the 1990s.[38,39] In these cases, immunogenicity was likely due to a new viral inactivation step introduced in the manufacturing of the FVIII products.[38] The i.v. N8-GP pathfinder clinical trial program includes five completed and two ongoing trials, with >270 patients treated with i.v. N8-GP, with >900 patient-years of

TABLE 4 Key immunogenicity results in part B

Patient	Inhibitor detected (+/−)	Anti-N8-GP binding antibodies detected (+/−)	Anti-N8-GP antibody–specific isotype detected (IgG4) (+/−)	FVIII trough levels affected (+/−)	FVIII mutation	Treatment–requiring bleeds during the trial including safety follow-up
A	+	+	+	+	Missense mutation	1
B	−	+	+	+	Intron 22 inversion	2
C	−	+	+	+	Small duplication	0
D	−	+	+	+	Intron 22 inversion	1
E	−	+	−	−	NR	4

Five patients developed anti-N8-GP antibodies in part B, four of whom tested positive for IgG4 antibodies. FVIII inhibitors were detected using the Nijmegen-modified Bethesda assay, including heat treatment of controls and samples. The presence of anti-N8-GP antibodies was determined by a validated radioimmunoassay and presence of antibodies confirmed in a confirmatory assay. Positive antibody samples were further characterized, eg, for immunoglobulin isotype and/or binding properties by a direct ELISA. +, inhibitor/antibodies detected; −, inhibitor/antibodies not detected. ELISA, enzyme-linked immunosorbent assay; FVIII, factor VIII; NR, not reported.

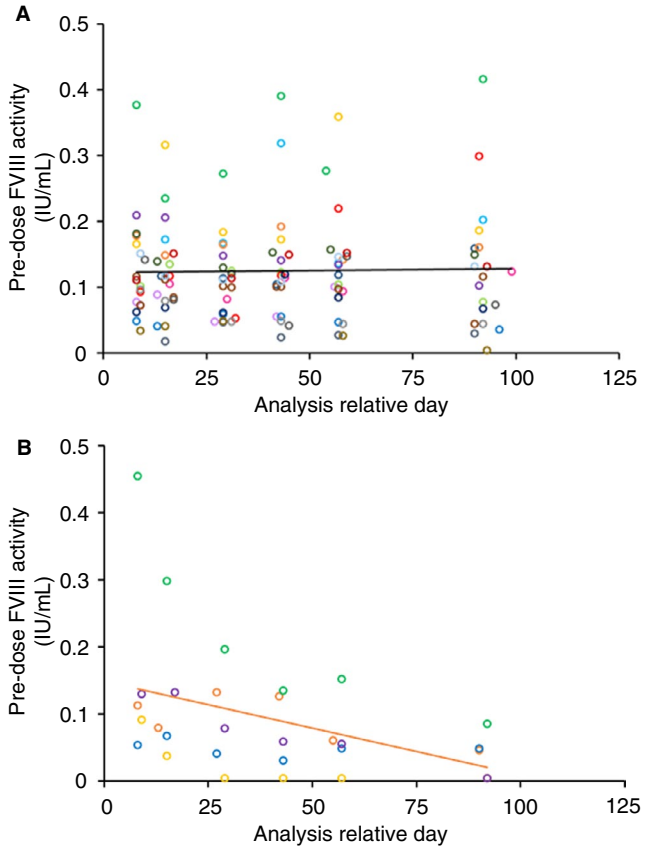

FIGURE 4 FVIII activity pre-dose versus time. Pre-dose FVIII activity measurements in individual patients without (A; n = 21) and with anti-N8-GP binding antibodies (B; n = 5) during part B. The pre-dose FVIII activity in patients with binding antibodies declined markedly over the course of the trial (B), which was not the case for patients who did not develop anti-N8-GP binding antibodies (A). FVIII, factor VIII

exposure and >5 years of clinical exposure in PTPs.[18,40] Only one PTP developed an inhibitor with i.v. N8-GP in these trials,[19-21,41] which suggests that the immunogenicity results in alleviate 1 are likely due to the s.c. method of administration, since the N8-GP molecules are otherwise identical. Subcutaneous administration exposes high concentrations of N8-GP to different components of the immune system compared with i.v. administration.[42] Furthermore, the transport of the N8-GP molecule into the vascular space via the lymphatic system may impact its immunogenicity.

Preclinical studies suggest that coagulation factor proteins administered subcutaneously are, potentially, more immunogenic than those administered intravenously. Considerably higher binding antibody titer levels were observed in hemophilia A mice following administration of s.c. FVIII compared with i.v. FVIII.[25] However, in a preclinical trial in which tolerance was induced with i.v. rFVIII in humanized hemophilia A mice, tolerance was not broken by changing the route of administration from i.v. to s.c.[43] These positive preclinical data supported the investigation of s.c. N8-GP in humans; however, preclinical data are not necessarily applicable to humans.

A possible reason for the increased immunogenicity associated with the s.c. route of administration is that the skin is a highly effective immunological organ that continually recognizes and eliminates a range of antigens.[44] Human skin contains a range of professional antigen-presenting cells, as well as the largest reservoir of T-cells in the body necessary to mount an immunologic response.[44-46] The late immunological response could be due to the instability of FVIII in s.c. tissue or possibly due to delayed and/or inefficient epitope spreading.[25]

The presence of high levels of anti-N8-GP binding antibodies correlated with declining FVIII activity in four patients. N8-GP-specific IgG4, which is known to correlate with inhibitor status,[47] was detected in four of the five patients with anti-N8-GP binding antibodies. AEs in part B were reported over a period of 6.46 patient-years of exposure; a longer duration of follow-up may have resulted in additional cases of antibody, or even inhibitor, development. Although it cannot be ruled out that a more sensitive assay would have detected a higher frequency of FVIII inhibitors, the incidence of clinically significant FVIII inhibitors in alleviate 1 was based on the threshold detection of ≥0.6 BU in accordance with World Federation of Hemophilia guidelines.[48] Nevertheless, the concern remained that these binding antibodies would have developed into FVIII inhibitors upon further exposure as indicated by the IgG maturation pattern and declining trough levels.

In contrast with the high incidence of anti-N8-GP binding antibodies in response to s.c. N8-GP, i.v. N8-GP was well tolerated in adults in the pathfinder 2 trial, without signs of increased immunogenicity.[20] These observations suggest that the s.c. route of administration of N8-GP increases its immunogenicity. Given the low rate of immunogenicity of i.v. N8-GP that was demonstrated in the pathfinder trials[19-21,41]—similar to that established for other intravenously administered FVIII concentrates[49,50]—it is highly plausible that the observed break of tolerance was due to the s.c. route of administration.

A potential limitation of this trial is that, like other trials of recombinant and modified FVIII molecules, the modified Bethesda assay for detection of inhibitors was used with the assumption that the inhibitory effect on plasma FVIII is reflective of inhibition of rFVIII and glycoPEGylated rVIII. However, this assumption has not been tested in *in vitro* studies.

5 | CONCLUSION

In this first-in-human clinical trial to investigate s.c. administration of N8-GP in PTPs, single-dose dose linearity was supported, with steady-state FVIII activity levels close to 10%. Preliminary efficacy data may suggest a potential for s.c. glycoPEGylated rFVIII prophylaxis; s.c. N8-GP was well tolerated and few treatment-requiring bleeds were reported. However, 5 out of 26 patients (19%) developed anti-N8-GP binding antibodies, one of whom developed inhibitors to FVIII. The development of high levels of binding antibodies and anti-N8-GP-specific IgG4 caused a concomitant decline in FVIII activity. The findings in this study demonstrate that s.c. administration of N8-GP is associated with a higher incidence of antibodies in PTPs. Therefore, further clinical development of s.c. N8-GP has been suspended. Indeed, very few studies have had the opportunity to directly compare the immunogenicity of the same drug administered by i.v. and s.c.[51] This study demonstrated that s.c. administration appears to substantially increase immunogenicity. The "late" immunogenic response to s.c. N8-GP after 42-91 EDs indicates that extended monitoring for anti-drug antibodies may be necessary in future studies of FVIII products, particularly after s.c. administration.

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CONFLICT OF INTERESTS

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AUTHORS' CONTRIBUTIONS

R. Klamroth, C. Feistritzer, S. R. Lentz, and P. Chowdary were primary study investigators and were involved in the conduct of the trial. K. Reichwald, U. Friedrich, and M. Zak were involved in the trial design, trial protocol, conduct of the trial, and data analysis. All authors contributed to the writing and editing of the manuscript.

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