

# An overview of turoctocog alfa pegol (N8-GP; ESPEROCT<sup>®</sup>) assay performance: Implications for postadministration monitoring

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## Abstract

Factor replacement therapy with factor VIII (FVIII) concentrates is the current standard of care for patients with haemophilia A. Postadministration monitoring of FVIII activity during on-demand or prophylactic treatment is important, for example to guide a suitable dosing regimen. While the use of two-stage chromogenic substrate (CS) assays is increasing, activated partial thromboplastin time (APTT)-based one-stage clotting (OSC) assays are most commonly used to measure FVIII activity in clinical laboratories. Substantial variations in activity measurements have been observed in association with some OSC assay reagents when assessing extended half-life FVIII molecules. Certain silica-based APTT reagents have previously been shown to underestimate FVIII activity with the polyethylene glycol (PEG)-conjugated product turoctocog alfa pegol (N8-GP [ESPEROCT<sup>®</sup>]; Novo Nordisk A/S). As a wide range of assay reagents are used in clinical laboratories worldwide, it is essential to establish which can be used to accurately measure activity with modified FVIII concentrates. Here, we describe the approach taken by Novo Nordisk to determine the suitability and accuracy of assays and reagents to measure FVIII activity in samples that contain N8-GP. While accurate activity measurements were possible with all tested CS assays and most of the OSC APTT reagents tested, three APTT reagents that contain silica as a contact activator were found to underestimate N8-GP recovery (APTT-SP, TriniCLOT<sup>™</sup>, STA<sup>®</sup> PTT-Automate). The data demonstrate the importance of characterizing the accuracy of each FVIII activity assay. Any limitations should be communicated to treating physicians and the clinical laboratories that test samples containing N8-GP.

## KEYWORDS

chromogenic substrates, drug monitoring, factor VIII, haemophilia A, partial thromboplastin time, turoctocog alfa pegol

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## 1 | INTRODUCTION

Haemophilia A is an X-chromosome-linked, congenital bleeding disorder caused by the deficiency or malfunction of coagulation factor VIII (FVIII). The current standard of care for patients with haemophilia A involves factor replacement therapy with intravenous recombinant or plasma-derived FVIII concentrates.<sup>1</sup> Accurate post-administration monitoring of FVIII activity is required to guide an appropriate dosing regimen during on-demand or prophylactic treatment. FVIII monitoring is most commonly performed using activated partial thromboplastin time (APTT)-based one-stage clotting (OSC) assays; two-stage chromogenic substrate (CS) assays are also used, albeit less frequently in clinical laboratory practice.<sup>2</sup> Although OSC assays have been traditionally used for clinical monitoring, emerging knowledge about their limitations has resulted in many laboratories adopting CS assays in recent years.<sup>3</sup>

The introduction of extended half-life recombinant FVIII (rFVIII) products has presented some challenges for clinical laboratories that use OSC assays to monitor the activity of unmodified FVIII products.<sup>4,5</sup> Substantial differences in inter-laboratory test results using some assays to monitor extended half-life molecules have been observed, a challenge that is compounded by the wide variety of reagents, instrumentation and methodology in use worldwide.<sup>2,6,7</sup> Variations in assay results appear to be related to the type of contact activator used in the APTT reagent, with some silica-based APTT reagents underestimating the activity of extended half-life rFVIII molecules.<sup>6,8-12</sup> Underestimation of FVIII activity is not desirable as it would add to treatment cost and potentially put patients at risk of thrombosis if the dosage is adjusted based on the result of the assay.<sup>5</sup> It is therefore important to identify assays that can be used to accurately monitor clinical postadministration samples and to communicate information regarding the most appropriate assays to use for monitoring to the clinical laboratories that perform these tests.

Turoctocog alfa pegol (N8-GP [ESPEROCT<sup>®</sup>]; Novo Nordisk A/S) is a polyethylene glycol (PEG)-glycoconjugated rFVIII molecule that was developed to extend the circulation half-life of turoctocog alfa (N8 [NovoEight<sup>®</sup>]; Novo Nordisk A/S).<sup>10</sup> In clinical trials, N8-GP has been shown to be an efficacious and well-tolerated treatment for children, adolescents and adults with haemophilia A.<sup>13-15</sup> N8-GP can be administered every 4th day, reducing the burden of frequent prophylactic treatment.<sup>13</sup>

Some OSC assay reagents have been demonstrated to underestimate the activity of N8-GP.<sup>8-12</sup> In this review, we give an overview of the programme undertaken by Novo Nordisk to characterize the functionality of N8-GP and examine the precision and accuracy of laboratory assays used to measure FVIII activity. We review data collected during the non-clinical and clinical programmes and provide guidance for clinical laboratories to assist in the selection of appropriate reagents to measure N8-GP activity.

## 2 | GLYCOPEGYLATION DOES NOT ALTER THE BIOLOGICAL ACTIVITY OF N8-GP

The conjugation of PEG to biological therapeutics has proven to be a safe and effective method with which to extend circulation half-life.<sup>16</sup> During the development of N8-GP, site-directed glycoPEGylation was used to attach a single branched 40-kDa PEG moiety to an O-linked glycan within the truncated FVIII B-domain of turoctocog alfa.<sup>10</sup> This B-domain and the attached PEG moiety are released after cleavage by thrombin, leaving an activated rFVIII molecule that is structurally identical to its unmodified counterpart. Data from clinical trials have shown that the half-life of N8-GP was extended 1.6-fold in adults and 1.9-fold in children when compared with the patients' previous (unmodified) FVIII products.<sup>14,15</sup>

**TABLE 1** VWF binding, rate of activation and cofactor function of N8-GP and N8

Parameters	N8-GP	N8
$K_d$ for VWF binding (nmol/L), n = 3	0.30 ± 0.03	0.20 ± 0.02
Rate of thrombin-mediated FVIII activation with 50 nmol/L VWF ( $\times 10^{-3}$ min <sup>-1</sup> ), n = 5	17.7 ± 2.8	14.7 ± 1.3
Rate of thrombin-mediated FVIII activation without VWF ( $\times 10^{-3}$ min <sup>-1</sup> ), n = 5	5.3 ± 3.6	4.0 ± 0.5
$K_{\frac{1}{2}\text{FIXa}}$ of FVIIIa binding to FIXa (nmol/L), n = 12-13	0.94 ± 0.13	0.78 ± 0.07
$K_m$ of FX activation (nmol/L), n = 10	4.9 ± 0.2	5.1 ± 0.5
$K_{\text{cat}}$ of FX activation (s <sup>-1</sup> ), n = 10	5.0 ± 1.5	6.5 ± 2.2
Rate of FVIIIa inactivation by APC (min <sup>-1</sup> ), n = 10	0.22 ± 0.04	0.23 ± 0.04

Note: Values are mean and SD noted from unpublished experiments conducted by the authors combined with data from Stennicke et al.<sup>10</sup> The rate of activation and cofactor function of N8-GP and N8 were analysed using enzyme kinetics and the binding to VWF assessed by ELISA.

Abbreviations: APC, activated protein C; ELISA, enzyme-linked immunosorbent assay; FIXa, activated factor IX; FVIII(a), (activated) factor FVIII; FX(a), (activated) factor X;  $K_{\frac{1}{2}\text{FIXa}}$ , apparent affinity of FIXa for FVIIIa;  $K_{\text{cat}}$ , turnover number;  $K_d$ , dissociation constant;  $K_m$ , Michaelis constant; N8, turoctocog alfa; N8-GP, turoctocog alfa pegol; SD, standard deviation; VWF, von Willebrand factor.

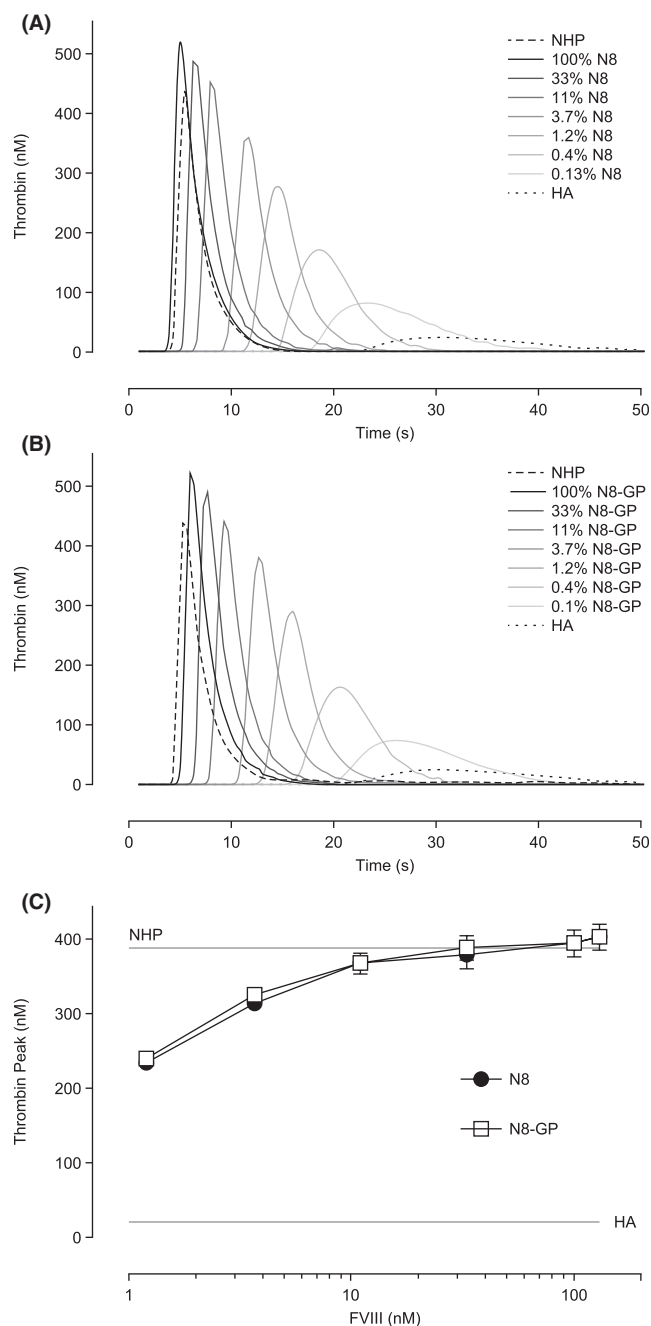
In order to demonstrate that the presence of the PEG moiety does not impact the functionality of N8-GP, experiments were designed to compare it with an unmodified rFVIII molecule (ADVATE® [Takeda] and N8). Biochemical data collected during the development process confirmed that binding to von Willebrand factor (VWF), rate of activation, cofactor functions and rate of inactivation of activated FVIII (FVIIIa) by activated protein C (APC) were not changed by glycoPEGylation in a biologically significant manner (Table 1).<sup>10</sup> While it appears that VWF binding affinity is lower with N8-GP, the dissociation constant ( $K_d$ ) values for both proteins are in the sub-nanomolar range. Assuming an N8 or N8-GP concentration of 1 IU/mL (0.625 nmol/L) and a VWF monomer plasma concentration of 30 nmol/L, a  $K_d$  value of 0.2 nmol/L will result in 99.3% of N8 binding to VWF, and a  $K_d$  value of 0.3 nmol/L will result in 99.0% of N8-GP binding to VWF. GlycoPEGylation did not influence the rate of activation of N8-GP compared with N8, both in the absence and presence of VWF. After activation, the kinetic parameters of N8-GP were comparable with N8, demonstrating that the cofactor functions were similar for both molecules.

Maintenance of biological activity after glycoPEGylation was furthermore confirmed by studies using the thrombin generation assay (TGA). Addition of N8-GP or N8 to plasma from patients with severe haemophilia A resulted in a comparable concentration-dependent response in the activated factor XI-triggered TGA (Figure 1A,B). At normal activity levels (100 IU/dL), the thrombin peaks for both N8-GP and N8 were similar to the thrombin peaks obtained using normal human plasma (Figure 1A,B). Thrombin peak values determined at a range of N8-GP and N8 concentrations were also congruent for both molecules (Figure 1C).

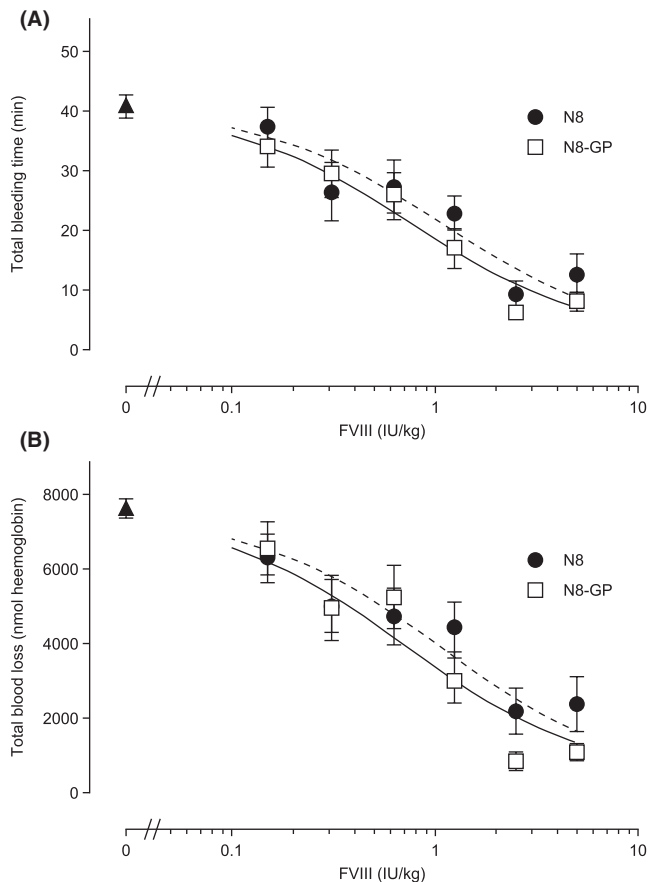
In vivo experiments were conducted with bleeding models using FVIII-deficient mice.<sup>17</sup> Dose-response curves for total bleeding time and total blood loss after tail-vein transection were shown to be comparable for N8-GP and N8, indicating that haemostatic effect is not affected by glycoPEGylation (Figure 2). These data were confirmed in another experiment in which N8-GP was compared with ADVATE® in the tail clip bleeding model.<sup>10</sup> Collectively, these results confirm that the presence of the PEG moiety does not impact the biological activity of N8-GP.

### 3 | N8-GP POTENCY LABELLING

Potency testing of clotting factor concentrates is used to assign each production lot with a specific potency value.<sup>18</sup> The potency of N8-GP is assigned according to European Pharmacopoeia (Ph. Eur.) recommendations<sup>19</sup> using a chromogenic assay (Coamatic® FVIII [Instrumentation Laboratory]). Assays are conducted with an in-house reference standard that has the same composition as N8-GP and is traceable to the World Health Organization (WHO) 8th international standard for FVIII concentrate (National Institute of Biological Standards and Control Code: 07/350). A study examining six CS assay kits (Coamatic® FVIII, Coatest® SP FVIII, Biophen™



**FIGURE 1** Effect of N8-GP in the TGA. The TGA monitors thrombin generation and decay in plasma samples after activation of coagulation by the addition of FXIa and phospholipids. The amount of thrombin in the reaction is quantified by measuring the cleavage of a thrombin-specific fluorogenic substrate. N8 (A) and N8-GP (B) at the concentrations noted were added to plasma from patients with severe haemophilia A, and thrombin generation was initiated with FXIa as described in Waters et al.<sup>25</sup> C, The effect of increasing N8 or N8-GP activity levels on peak thrombin values triggered by FXIa. Grey horizontal lines represent peak values obtained with NHP and HA. Data are the mean  $\pm$  SEM ( $n = 12$ ).<sup>25</sup> Reprinted with permission.<sup>25</sup> FVIII, factor VIII; FXIa, activated factor XI; HA; haemophilia A plasma; N8, turoctocog alfa; N8-GP, turoctocog alfa pegol; NHP, normal human plasma; SEM, standard error of the mean; TGA, thrombin generation assay



**FIGURE 2** In vivo evaluation of N8-GP activity in FVIII knockout mice. Dose-response curves for total bleeding time (A) and total blood loss (B) were determined for N8 and N8-GP-treated mice (dotted line: N8; black line: N8-GP) in a tail-vein transection bleeding model.<sup>17</sup> Five minutes after intravenous dosing, left lateral tail-vein transection was performed on anesthetized FVIII-deficient mice ( $n = 12$  per time point). Data points show mean  $\pm$  SEM. Reprinted with permission.<sup>17</sup> FVIII, factor VIII; N8, turoctocog alfa; N8-GP, turoctocog alfa pegol; SEM, standard error of the mean

FVIII:C, Technochrom<sup>®</sup> FVIII:C, DG-Chrom FVIII and Siemens FVIII Chromogenic Assay [Supplier information for all listed assay reagents can be found in Table 2]) adapted to Ph. Eur. guidelines showed consistent and accurate measurements of FVIII activity for potency testing and clinical postadministration monitoring of N8-GP.<sup>18</sup>

#### 4 | POSTADMINISTRATION MONITORING OF N8-GP

While N8-GP was in development, it was noted that the specific activity of N8-GP varied when using certain APTT reagents in OSC assays.<sup>10</sup> Tiefenbacher et al<sup>12</sup> also highlighted discrepancies in activity measurements of N8-GP associated with a single silica-based APTT reagent.<sup>9,12</sup> While acceptable recoveries were achieved with Actin<sup>®</sup> FSL and CK Prest<sup>®</sup> APTT reagents, the silica-based STA<sup>®</sup> PTT-Automate reagent significantly underestimated N8-GP activity.<sup>9,12</sup> Additionally, a two-centre study assessed a number of different OSC

and CS assays to measure N8-GP activity in spiked samples compared with an unmodified rFVIII (ADVATE<sup>®</sup>).<sup>8</sup> Acceptable N8-GP recovery, defined as  $\pm 30\%$  of target, was observed with five APTT reagents tested at both centres (Actin<sup>®</sup> FS, Pathromtin<sup>®</sup> SL, DG Synth, SynthAFax<sup>®</sup> and SynthASil<sup>®</sup>). A single silica-based reagent, APTT-SP, considerably underestimated N8-GP activity when compared with unmodified FVIII (Figure 3). Target recoveries obtained using APTT-SP ranged between 30% and 50% across both sites.<sup>8</sup> All three CS assay kits evaluated (Siemens FVIII Chromogenic Assay, Coamatic<sup>®</sup> FVIII and Coatest<sup>®</sup> SP FVIII) recovered the activity of N8-GP accurately.<sup>8</sup>

To further examine the accuracy of N8-GP activity measurements in the real world, a large field study was conducted during which clinical laboratories used their routine FVIII activity assays to test samples spiked with four different levels of N8-GP or unmodified rFVIII (ADVATE<sup>®</sup>) in the range of 0.03–0.9 IU/mL.<sup>11</sup> The actual potencies of both N8-GP and ADVATE<sup>®</sup> vials were used in the preparation of field study kits and were based on a chromogenic assay according to Ph. Eur. guidelines. A total of 67 laboratories in 25 countries participated in the study; among these, 60 (90%) used OSC assays and 36 (54%) used CS assays (29 [43%] used both; Figure 4A). Among a total of nine APTT reagents tested, acceptable measurement of N8-GP activity was achieved using six of the most commonly used reagents (CK Prest<sup>®</sup>, SynthASil<sup>®</sup>, Pathromtin<sup>®</sup> SL, Actin<sup>®</sup> FS, Actin<sup>®</sup> FSL and APTT-SLA; Figure 4B). Mean recoveries were 92.5% of target concentration for N8-GP and 123% for ADVATE<sup>®</sup>, where acceptable recovery was defined as  $\pm 30\%$ . The threshold value of  $\pm 30\%$  has been used in previous studies and was chosen based on published assay variability data.<sup>20,21</sup> In agreement with previous results, three silica-based APTT reagents (APTT-SP, TriniCLOT<sup>™</sup> and STA<sup>®</sup> PTT-Automate) were shown to underestimate N8-GP recovery. Although TriniCLOT<sup>™</sup> had an acceptable recovery of 83% at an N8-GP concentration of 0.03 IU/mL, recoveries at higher concentrations were not in the acceptable range (40%–48%). APTT-SP and STA<sup>®</sup> PTT-Automate measured N8-GP activity at 43%–60% of the target for each N8-GP concentration tested. Among a total of 33 European laboratories surveyed, only two used APTT-SP (Belgium and The Netherlands) and three used TriniCLOT<sup>™</sup> (Denmark, France and the UK). In the United States, only one out of eight laboratories used one of the reagents that underestimated activity (STA<sup>®</sup> PTT-Automate). All of the six CS assay kits evaluated (Coamatic<sup>®</sup> FVIII, Coatest<sup>®</sup> SP FVIII, Biophen<sup>™</sup> FVIII:C, Technochrom<sup>®</sup> FVIII:C, Siemens FVIII Chromogenic Assay and Electrachrome<sup>™</sup> FVIII) showed acceptable recovery of N8-GP (Figure 4C). Mean recoveries were 129% of target concentration for N8-GP and 127% for ADVATE<sup>®</sup>.

#### 5 | ACTIVITY MEASUREMENTS WITH OTHER PEGYLATED rFVIII PRODUCTS

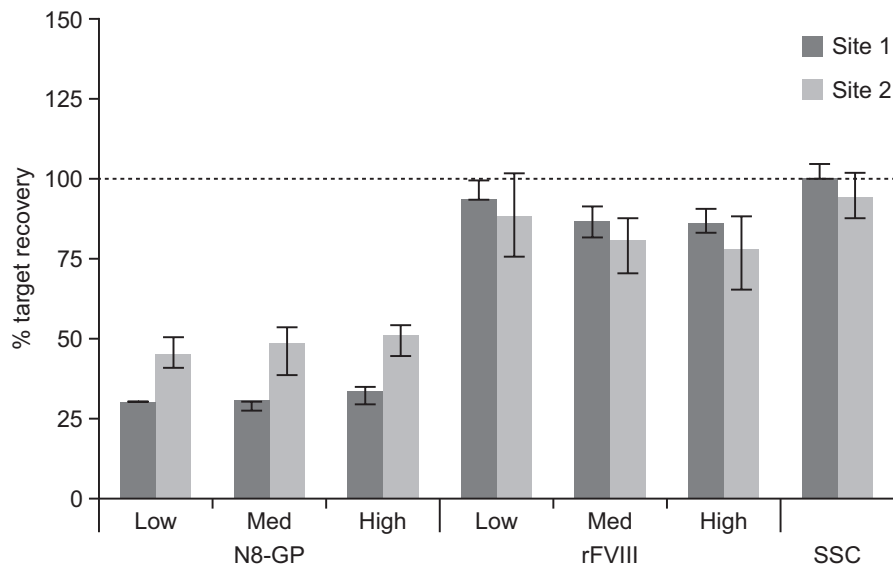
In a recent study by Church et al,<sup>6</sup> FVIII activity measurements for damoctocog alfa pegol (BAY 94 9027 [JIVI<sup>®</sup>]; Bayer) were underestimated at all concentrations when using the silica-based APTT-SP and



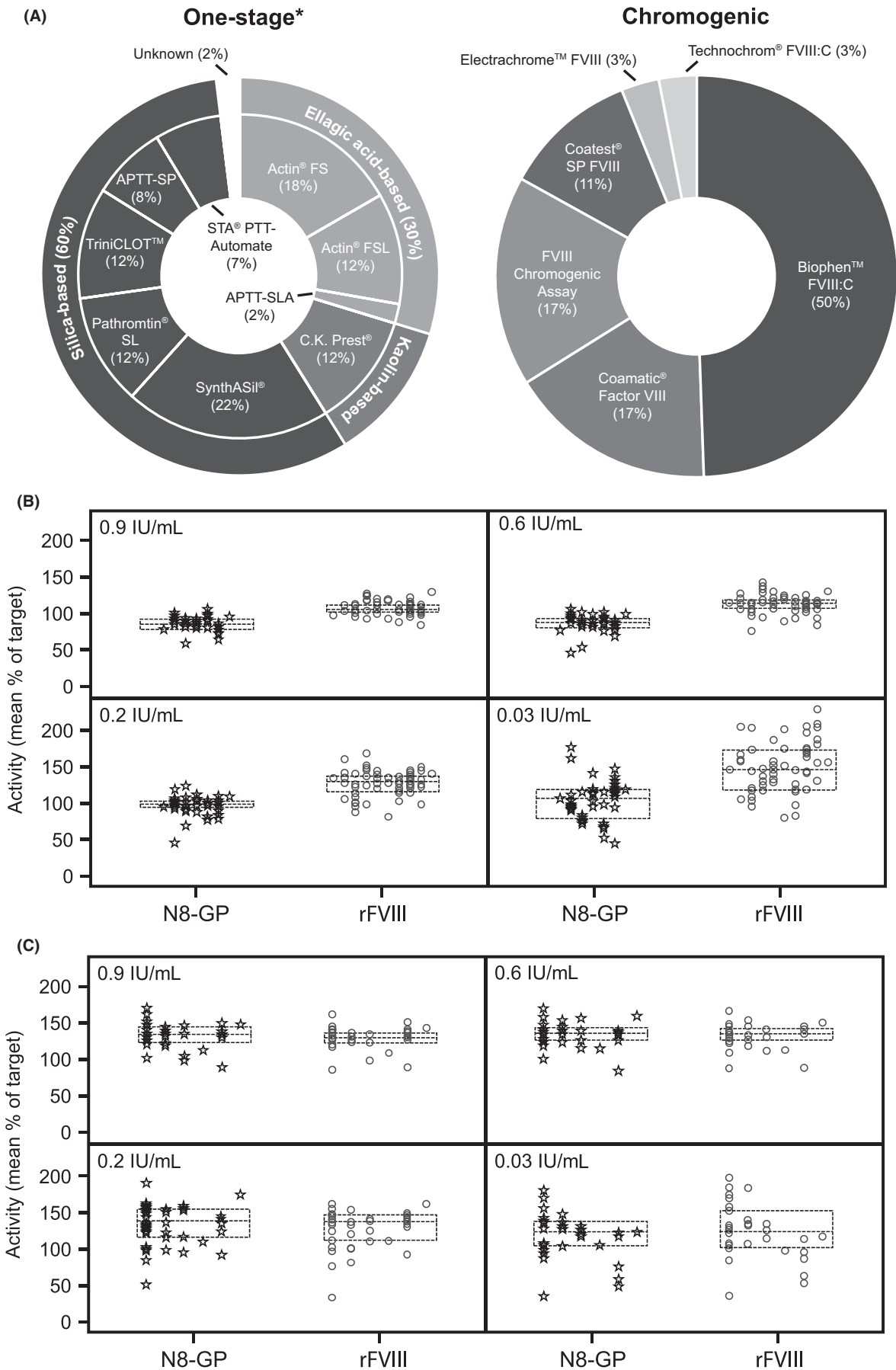
Assay	Activator	Supplier	Reference
Assays with acceptable recovery of N8-GP activity			
Chromogenic assays			
Siemens FVIII Chromogenic Assay	NA	Siemens Healthcare GmbH	8,11,18
Coamatic <sup>®</sup> FVIII	NA	Chromogenix	8,11,13-15,18
Coatest <sup>®</sup> SP FVIII	NA	Chromogenix	8,11,18
Biophen <sup>™</sup> FVIII:C	NA	Hyphen Biomed	11,18
Technochrom <sup>®</sup> FVIII:C	NA	Technoclone GmbH	11,18
Electrachrome <sup>™</sup> FVIII	NA	Instrumentation Laboratory	11
DG-Chrom FVIII	NA	Grifols	18
One-stage APTT assay reagents			
Actin <sup>®</sup> FS	Ellagic acid	Siemens Healthcare GmbH	8,11
Actin <sup>®</sup> FSL	Ellagic acid	Siemens Healthcare GmbH	9-12
APTT-SLA	Ellagic acid	Symex Corporation	11
Pathromtin <sup>®</sup> SL	Silicon dioxide	Siemens Healthcare GmbH	8,11,26
SynthASil <sup>®</sup>	Colloidal silica	Instrumentation Laboratory	8,11,14
SynthAFax <sup>®</sup>	Ellagic acid	Instrumentation Laboratory	8
DG Synth	Ellagic acid	Grifols	8
CK Prest <sup>®</sup>	Kaolin	Diagnostica Stago UK	9,11,12
Assays that underestimate N8-GP recovery			
One-stage APTT assay reagents			
APTT-SP	Colloidal silica	Instrumentation Laboratory	8,11
TriniCLOT <sup>™</sup>	Micronized silica	Diagnostica Stago UK	10,11
STA <sup>®</sup> PTT-Automate	Silica	Diagnostica Stago UK	9-12

**TABLE 2** Assays assessed for their ability to recover N8-GP FVIII activity

Abbreviations: APTT, activated partial thromboplastin time; FVIII, factor VIII; NA, not applicable; N8-GP, turoctocog alfa pegol.



**FIGURE 3** Comparison of N8-GP and rFVIII activity measured by one-stage clotting assay at two sites using the APTT-SP reagent. Site 1 was located in Sheffield, UK, and site 2 was located in Malmö, Sweden. FVIII:C was determined in severe haemophilia A plasma samples spiked with low (0.20 IU/mL), medium (0.60 IU/mL) or high (0.90 IU/mL) concentrations of N8-GP or rFVIII (ADVATE<sup>®</sup>). Measurements were performed in three independent replicates. Samples were analysed in three dilutions, and all assays passed local parallelism checks indicating satisfactory parallelism between samples and reference standards. Data are presented as the percentage of target recovery and error bars show range ( $\pm$ ). The dotted line represents a target recovery of 100%. Acceptable N8-GP recovery was defined as  $\pm 30\%$  of target. Reprinted with permission.<sup>8</sup> FVIII, factor VIII; FVIII:C, FVIII activity; N8-GP, turoctocog alfa pegol; rFVIII, recombinant factor VIII; SSC, Scientific and Standardization Committee plasma samples







**FIGURE 4** Results from the N8-GP field study. A, Overview of methods used for FVIII activity measurement. For OSC assays, the type of contact activator is shown in the outer circle, and the APTT reagent is shown in the inner circle. B, C, Mean FVIII:C values for N8-GP and rFVIII (ADVATE<sup>®</sup>) measured using OSC (B) and CS (C) assays in 67 individual laboratories. Dotted lines show interquartile range. Three agents that underestimated N8-GP activity were omitted from statistical analyses (APTT-SP, TriniCLOT<sup>™</sup> and STA<sup>®</sup> PTT-Automate). Reprinted with permission.<sup>11</sup> \*Some laboratories reported the use of multiple FVIII:C measurement methods. (r)FVIII, (recombinant) factor VIII; APTT, activated partial thromboplastin time; CS, chromogenic substrate; FVIII:C, factor VIII activity; N8-GP, turoctocog alfa pegol; OSC, one-stage clotting

STA<sup>®</sup> PTT-Automate reagents. This is in agreement with results described above for N8-GP. However, in the same study, Actin<sup>®</sup> FS and CK Prest<sup>®</sup> reagents showed some overestimation of BAY 94 9027 activity at low concentrations.<sup>6</sup> In comparison, no overestimation of FVIII activity has been observed for N8-GP. Significantly prolonged clotting time ( $P < .001$ ), that is reduced FVIII activity, was also observed by Gu et al<sup>7</sup> when APTT-SP and STA<sup>®</sup> PTT-Automate reagents were used to measure FVIII-deficient plasma spiked with BAY 94 9027 relative to the WHO 8th international FVIII reference standard.

## 6 | THE MECHANISM BEHIND UNDERESTIMATION OF FVIII ACTIVITY WITH CERTAIN SILICA-BASED APTT REAGENTS

A recent study showed that thrombin-induced activation of N8-GP proceeded more slowly in the presence of the silica-based APTT-SP reagent when compared with unmodified N8.<sup>22</sup> This was not the case for other non-silica-based APTT reagents. Both PEGylated and unmodified rFVIII products exhibited a similar degree of adsorption to silica particles, suggesting that contact activation may not be blocked through specific adherence of the PEGylated molecule to the silica surface. However, PEGylated and unmodified FVIII molecules may adsorb to silica in different orientations, which might make N8-GP less accessible to cleavage by thrombin than N8. Delayed activation may disrupt FVIIIa-dependent formation of activated factor X in plasma, prolonging clotting time and resulting in underestimation of N8-GP activity. While these findings illustrate that PEGylation appears to influence contact activation by silica, it is important to note that there are no clinical consequences of this modification as the efficacy of N8-GP has been demonstrated in a number of studies.<sup>13-15</sup>

## 7 | CONCLUSIONS AND RECOMMENDATIONS FOR N8-GP POSTADMINISTRATION MONITORING

A number of characterization analyses showed no effect of PEG on the biological and biochemical properties of N8-GP when compared to unmodified rFVIII molecules (N8 and ADVATE<sup>®</sup>). Experiments that examined the precision and accuracy of laboratory assays that may be used to measure the FVIII activity of N8-GP demonstrated that FVIII activity can be accurately measured using CS assays and the majority of available OSC assay APTT

reagents (Table 2). Three silica-based APTT reagents (APTT-SP, TriniCLOT<sup>™</sup> and STA<sup>®</sup> PTT-Automate) underestimate N8-GP recovery.<sup>8,11</sup> None of the tested reagents were shown to overestimate activity, and thus, there is no risk of underdosing due to assay discrepancies. Clinical laboratories should be made aware of the assay variability associated with a few APTT reagents used for postadministration monitoring of haemophilia A patients treated with certain modified rFVIII molecules. This information has been highlighted in the US Food and Drug Administration (FDA) package insert and the European Medicines Agency (EMA) summary of product characteristics for N8-GP.<sup>23,24</sup> As the availability of some reagents may be country- or region-specific, the accuracy of locally marketed APTT reagents, particularly those that use silica as a contact activator, should be tested individually.

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### DISCLOSURES

All authors are current or former employees of Novo Nordisk A/S and are minor Novo Nordisk A/S shareholders.

### AUTHOR CONTRIBUTIONS

ME conceived and outlined the manuscript. All authors have made substantial contributions to the interpretation of the contents and the drafting of the manuscript, and have approved the final version.

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