OPEN

Evaluation of recombinant factor VIII Fc (Eloctate) activity by thromboelastometry in a multicenter phase 3 clinical trial and correlation with bleeding phenotype

Frank Driessler[a], Maricel G. Miguelino[b], Glenn F. Pierce[a], Robert T. Peters[a] and Jurg M. Sommer[a]

The aim of this study was to compare the hemostatic efficacy of recombinant factor VIII Fc (rFVIIIFc) (Eloctate) and Advate by ex-vivo rotation thromboelastometry (ROTEM) of whole blood and to explore potential ROTEM parameters that may be more predictive of a patient's bleeding tendency than plasma FVIII activity. Thirteen clinical sites were selected to perform ROTEM on freshly collected blood samples from 44 patients in the phase 3 study for rFVIIIFc, including 16 patients undergoing sequential pharmacokinetic assessment of Advate and rFVIIIFc. Equivalent hemostatic activity was observed for rFVIIIFc and Advate in postinfusion samples, followed by improvements for rFVIIIFc in clotting time, clot formation time and alpha angle (α) for a longer duration than Advate, consistent with the pharmacokinetic improvements reported previously for rFVIIIFc. Our study did not demonstrate a statistical correlation between a patient's ROTEM activity at baseline or at trough and the occurrence of spontaneous bleeds while on prophylactic therapy. However, an association was observed between postinfusion clotting time and the occurrence of one or more spontaneous bleeds vs. no bleeds over a follow-up period of 1 year ($P = 0.003$). How well a patient's whole blood clotting deficiency is corrected after a dose of FVIII may be an indicator of subsequent bleeding tendency in patients with otherwise equivalent FVIII peak and trough levels. The technical challenges of standardizing the ROTEM, largely overcome in the current study, may however preclude the use of this method for widespread assessment of global hemostasis unless additional assay controls or normalization procedures prove to be effective.

Blood Coagulation and Fibrinolysis 2017, **28**:540–550

Keywords: clinical trial, Eloctate, factor VIII, global hemostasis, hemophilia, recombinant factor VIII Fc, thromboelastography, thromboelastometry

[a]Bioverativ, Waltham, Massachusetts and [b]UC Davis Medical Center, Sacramento, California, USA

Correspondence to Jurg M. Sommer, Bioverativ, 225 Second Ave., Waltham, MA 02451, USA
Tel: +1 781 663 2868; e-mail: jurg.sommer@bioverativ.com

Received 2 February 2017 **Revised** 27 March 2017
Accepted 10 April 2017

Introduction

Rotational thromboelastometry (ROTEM) is an enhancement of the classical thromboelastography (TEG), a test for the assessment of global hemostasis by continuous recording of whole blood clot formation, clot characteristics and clot degradation [1]. ROTEM and TEG are primarily used to monitor hemostasis during surgical procedures [2] and evaluate the procoagulant activity of bypass agents in the treatment of hemophilia with inhibitors [3,4]. A significant fraction of persons with severe hemophilia A, defined as having less than 1% of normal plasma factor VIII (FVIII) activity, do not present with spontaneous bleeding patterns typically associated with this group. Conversely, some persons with moderate hemophilia (1–5% FVIII) exhibit a bleeding phenotype more severe than expected on the basis of their levels of endogenous FVIII activity. The reason for these discrepancies is not well understood but could in part be because of the significant biological variability among other coagulation factors [5–7]. Laboratory assays that represent the entire coagulation process might predict the individual bleeding risk more accurately than FVIII activity and such a test would offer considerable clinical benefits by optimizing treatment regimens [8]. By measuring the hemostatic potential in a patient's whole blood sample, ROTEM and TEG may thus provide clinically relevant information on a patient's hemostasis that cannot be obtained from a plasma-based clotting assay.

Conceptually, clot formation may be considered as three overlapping phases: initiation, amplification and clot propagation. In this model, the coagulation defect in hemophilia A is thought to be in the amplification phase in which large-scale thrombin generation occurs. This phase is required for effective fibrin production and clot propagation [9]. As such, one might expect the differences in residual hemostatic activity between hemophilia A patients to be primarily a function of CT [the clotting time from recalcification to an amplitude (clot firmness) of 2 mm], which is thought to encompass the clot initiation and amplification phases. CFT (clot formation time from CT until a clot firmness of 20 mm) and α angle (angle of the tangent to the curve at a 2-mm amplitude) reflect primarily the clot propagation phase, which is

0957-5235 DOI:10.1097/MBC.0000000000000638

largely influenced by platelet function and fibrinogen levels [10]. With careful titration of the coagulation activators, residual endogenous FVIII activity well below 1 IU/dl can be detected by ROTEM or TEG, which has also been proposed to account for interpatient differences in severe hemophilia [10,11].

The lack of standardized methods and suitable reference material precludes comparison of data between laboratories; ROTEM and TEG have therefore undergone limited application in assessing or predicting FVIII and factor IX clinical responses. Several studies have nevertheless attempted to correlate a patient's bleeding phenotype with global hemostasis parameters in the hope of improving individualized patient care [12–18]. Although increasing levels of thrombin generation and whole blood clotting activity can be observed in patients with severe, moderate or mild hemophilia, a statistical association between global hemostasis measurements and clinical phenotype within the severe hemophilia population has been difficult to establish.

Recombinant FVIII Fc fusion protein (rFVIIIFc; Eloctate; Bioverativ, Cambridge, Massachusetts, USA) is the first extended half-life (EHL) FVIII product approved for the control and prevention of bleeding episodes and perioperative management in adults and children with hemophilia A. The Fc domain of human IgG_1 enables the fusion protein to bind to the neonatal Fc receptor, part of an endogenous intracellular pathway that delays lysosomal degradation of Fc-containing proteins (i.e. IgG) by cycling them back into circulation [19]. The phase 3 'A-LONG' study for evaluation of the safety, pharmacokinetics and efficacy of rFVIIIFc in previously treated patients with severe hemophilia A demonstrated an extended plasma half-life of rFVIIIFc relative to Advate (~1.5-fold increase, 19.0 h), as well as the safety and efficacy of rFVIIIFc for the control and prevention of bleeding episodes [20,21].

Potency of rFVIIIFc was assigned against the WHO 8th International Standard by a validated FVIII chromogenic substrate assay. One stage clotting and chromogenic activities of rFVIIIFc differ by less than 20% [22], and a field study in 30 clinical hemostasis laboratories demonstrated that the activity of rFVIIIFc in spiked samples could be measured by all commonly used one stage clotting assays with accuracy and variability similar to that of Advate. Significantly higher reagent-dependent discrepancies were observed for other EHL FVIII products [23], and some uncertainty remains whether the chosen method of potency assignment for modified replacement factors results in hemostatic efficacy in patients that is equivalent to that of conventional FVIII products.

The primary aim of this ROTEM study was to confirm equivalent ex-vivo whole blood clotting activity in post-infusion samples collected from patients receiving Advate and rFVIIIFc. We also explored a number of possible links between ROTEM activity and bleeding tendency in an attempt to uncover statistically meaningful correlations. It is important to note, however, that A-LONG study patients experienced relatively few bleeds, if any, and this study was not prospectively designed to evaluate the correlation between ROTEM results and bleeding frequency.

Methods

Clinical study design

The A-LONG study was a phase 3 open label, multicenter, partially randomized study of rFVIIIFc in patients with severe hemophilia A. The protocol was approved by local institutional review boards and ethics committees, and the study was conducted in accordance with the International Conference on Harmonization Guidelines for Good Clinical Practice and the ethical principles outlined in the Declaration of Helsinki. All patients, or patient guardians, gave written informed consent. The study was registered with ClinicalTrials.gov, number NCT01181128.

Rotation thromboelastometry site selection and training

Study sites were chosen on the basis of the investigator's interest, existing ROTEM instrument and expertise among the 60 centers participating in the A-LONG study. Both ROTEM instrument models Gamma and Delta were used in this study, and no differences were observed for the quality control (QC) samples. All 13 participating sites were visited by a Bioverativ scientist at least once prior to study initiation to review the procedure and provide hand-on training.

Reagents and analytical controls

Each site was provided with a detailed assay procedure, all required reagents and frozen plasma controls to ensure consistent assay performance. Unique lots of star-tem, in-tem and recombinant (*r*) ex-tem reagents were reserved for this study to ensure that every site used the same reagent lots. These reagents were obtained from Tem Innovations GmbH and distributed to the participating sites by Bioverativ. The in-tem and ex-tem activator reagents were titrated for optimal sensitivity to FVIII across the entire pharmacokinetic range, and all sites prepared identical dilutions of these reagents by using prefilled vials of dilution buffer provided by Bioverativ. ROTEM instruments were calibrated by the vendor and performed within specifications as demonstrated by the manufacturer's 'ROTROL N' control (data not shown). Additional plasma controls prepared by Bioverativ, which contained Advate spiked into congenital FVIII-deficient plasma at 1, 5, 15 and 30 IU/dl, allowed a more detailed analysis of instrument and operator performance across the sites. These plasma control samples were tested in the INTEM, NATEM and EXTEM assays at each site

1–2 days prior to the analysis of a series of pharmacokinetic samples.

Sample collection

All sample testing was performed locally using whole blood freshly collected into 3-ml vacutainers containing 0.109-mol/l sodium citrate. Time points for blood collection coincided with the sampling schedule of the pharmacokinetic assessment(s) for each patient. All dosing during pharmacokinetic assessments were based on actual vial potency rather than nominal activity. Prior to Advate or rFVIIIFc infusion, patients had a washout period of at least 96 h. In the sequential Advate + rFVIIIFc subgroup, samples for ROTEM analysis were collected predose, 6 (±1) h, 24 (±2) h (Day 1), 48 (±2) h (Day 2) and 72 (±2) h (Day 3) from the start of the Advate infusion. During the subsequent rFVIIIFc pharmacokinetic assessments at 'Week 1' and 'Week 14', ROTEM samples were collected predose, 6 (±1) h, 24 (±2) h (Day 1), 72 (±2) h (Day 3), 96 (±2) h (Day 4), and 120 (±2) h (Day 5) from the start of the rFVIIIFc infusion. Thus, ROTEM analysis was performed at the same time points as the plasma FVIII activity determinations, with the exception of the immediate postinfusion (10 or 30-min) and 1-h pharmacokinetic time points. Samples from these two time points could not be analyzed as the run time for the baseline ROTEM sample lasted up to 3 h.

Analytical procedure

Citrated whole blood samples were kept undisturbed at ambient temperature until ROTEM analysis was initiated consistently between 30 and 45 min after blood collection. For the INTEM analysis, an intrinsic (contact) activator, the in-tem reagent (ellagic acid) was diluted 300-fold in 10-mmol/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer pH 7.4 prior to addition of 20–300 μl of citrated blood. In the EXTEM analysis, human recombinant tissue factor (TF) was added as an extrinsic activator by diluting the ex-tem reagent 10 000 fold in 10 mmol/l HEPES buffer pH 7.4 supplemented with 1 mg/ml human serum albumin to approximately 1-pM TF. The exact TF concentration was not determined. The samples were recalcified by addition of 20 μl star-tem reagent immediately before initiating the ROTEM. The INTEM, EXTEM and NATEM ('native' activation by recalcification using only the star-TEM reagent) (recalcification by star-tem reagent only) runs were performed in parallel. With the availability of a fourth channel on the ROTEM instrument, one duplicate run could be performed at the discretion of the operator. Sample analysis was continued for at least 2 h or until the tracing indicated that maximal clot firmness was reached, but no longer than 3 h.

Data management

Run data were stored electronically on the ROTEM instrument and exported into tab-deliminated Excel files (Microsoft Corporation, Redmond, WA, USA) specific for each patient and pharmacokinetic profile. The electronic files were transmitted to Bioverativ for analysis. In addition, worksheets were provided to the sites for manual capture of study-specific data including patient identity, date and time of assay, reagent information, QC results, as well as the results for four key parameters from each run [CT, CFT, α angle, and maximum clot firmness (MCF) by NATEM, INTEM and EXTEM]. QC data provided by each site were reviewed by Bioverativ to verify instrument and operator performance prior to clinical sample analysis. To ensure the integrity of the ROTEM data, SAS (Statistical Analysis Software, SAS Institute Inc., Cary, NC, USA) statistical software was used to verify accurate assignment of all runs to their respective patients and pharmacokinetic time points. In case both results from duplicate runs were valid, the average value was used in the analysis. For 163 of the 1379 ROTEM runs performed (of which 223 were duplicate sample analyses), the instrument issued an error code. Inspection of the raw data showed that 148 of the 163 error codes were not applicable to the four primary ROTEM parameters evaluated in this study. An additional 37 runs without an instrument error code produced invalid data for various reasons, including lack of data recording or outlier results, for example extremely low CT values, likely due to preanalytical mistakes (preactivation). In total, 52 runs produced invalid results. Eighteen of the 52 invalid runs had duplicate analyses that produced a valid result. Thus, the overall rate of ROTEM samples that were unanalyzable and/or for which the results were rejected was 2.9%.

Statistical analysis

Microsoft Excel and/or GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) were used for analysis and data plotting. Estimated CT values at trough and 'Time to 1000 s CT' were calculated assuming an exponential decay of FVIII activity and a log-linear relationship of clot time vs. FVIII activity at at least 6 h, thus resulting in a model of linear increase of CT over time. For patients that used an rFVIIIFc dose in routine prophylaxis that was different from the dose used in the pharmacokinetic and ROTEM assessments (50 IU/kg), the estimated CT values were adjusted on the basis of the individual's log (FVIII) vs. CT relationship derived from linear regression analysis.

Results

Rotation thromboelastometry assay standardization

Thirteen of the 60 A-LONG clinical sites participated in the ROTEM study. Standardization of the blood collection procedure and analytical method was an essential part of this study to ensure comparable results across all sites. Table 1 shows the INTEM results from 13 sites that performed a total of 31 control runs using shared frozen hemophilic plasma controls spiked at four different levels

Table 1 Performance of frozen plasma control samples (INTEM clotting time) at clinical sites compared with in-house (Bioverativ) results

	Clinical sites (13 sites, 31 runs)				Bioverativ site (4 instruments, 8 runs)			
FVIII concentration (IU/dl)	0.30	0.15	0.05	0.01	0.30	0.15	0.05	0.01
Mean CT (INTEM)	688.1	796.1	940.8	1368	702.1	779.0	1029	1462
SD	107.1	118.5	96.82	236.7	30.47	38.83	46.51	120.2
Coefficient of variation (% CV)	15.6	14.9	10.3	17.3	4.3	5.0	4.5	8.2

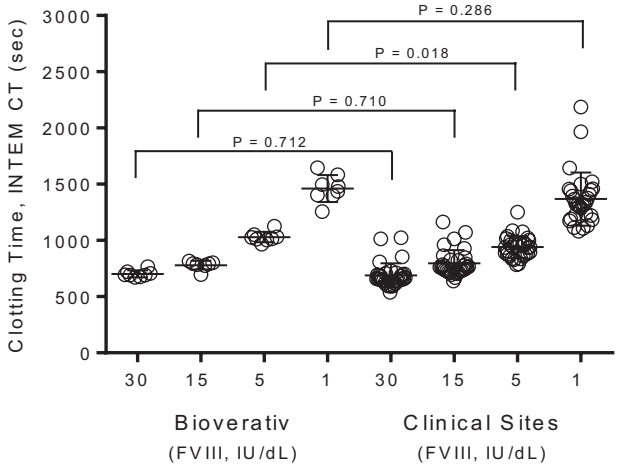
SD, Standard deviation.

of FVIII. The mean CTs for the plasma controls among all sites were not statistically different from the in-house measurements obtained during eight independent runs on four instruments by a single operator (Fig. 1). The variability in the clot time [INTEM % coefficient of variation, (CV)] for the thirteen sites ranged from 10.3 to 17.3% at the four FVIII levels, which was approximately twice the variability observed for repeated in-house measurements (4.3 to 8.2% CV). No outlier laboratories were observed. The average CV across all sites and FVIII concentrations (15% CV) was considered adequate for this study as the primary aim was to compare the hemostatic potential of Advate vs. rFVIIIFc within each patient, largely depending on assay precision at each site. Although these QC procedures were useful for verifying instrument performance, reagent quality and operator competence, they could not account for the variety of preanalytical variables that can potentially affect coagulation assays [24].

Baseline rotation thromboelastometry assessment

Forty-four A-LONG patients underwent ROTEM analysis, which was performed only during the time of their pharmacokinetic assessment. Twenty-six patients had no measurable FVIII activity (<0.5 IU/dl) in their ROTEM baseline sample prior to infusion of FVIII. Interpatient variability (% CV) for the INTEM CT in these samples was 42%. For 25 of 26 patients that formed a clot by INTEM in the absence of FVIII during the 2–3 h of measurement, the interpatient % CV for CFT and α angle were 85 and 27%, respectively. Twelve patients had no measurable FVIII activity on the final day(s) of the pharmacokinetic profiling and thus had two or more 'baseline' ROTEM samples. The average intrapatient variability for the INTEM CT in these patients was 22% (range 0.4–53%), whereas the interpatient variability for the mean CT was 25% in the same group. The observation that multiple ROTEM measurements per individual greatly reduced the interpatient variability, and the relative closeness of intrapatient and interpatient variability suggests that in our study, a single ROTEM measurement may not be a reliable marker for evaluating a patient's whole blood clotting potential at baseline. Residual or endogenous FVIII activity below 0.5 IU/dl, or preanalytical variability that may not have been well controlled across all sites, likely contributed to high ROTEM variability at baseline.

Fig. 1



INTEM clotting time of spiked plasma control samples (30, 15, 5 and 1 IU/dl of Advate) analyzed in 31 runs at 13 clinical sites are comparable with clotting times from eight independent runs performed on four instruments at Bioverativ. Bars show mean QC result ± SD.

Postinfusion and pharmacodynamic rotation thromboelastometry assessment

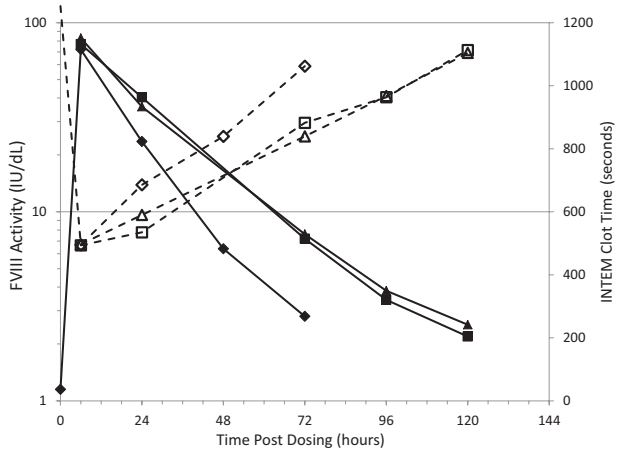
Sixteen of the 44 ROTEM patients were part of the pharmacokinetic subgroup that received both Advate and rFVIIIFc. The earliest sample after Advate or rFVIIIFc administration that could be evaluated in this subgroup was collected 6-h postinfusion (see the 'Methods' section), at which point comparable mean clot times were observed for Advate and rFVIIIFc (Table 2). Twenty-four hours after infusion, and at all subsequent time points, the samples collected from patients dosed with rFVIIIFc exhibited shorter whole blood CTs than samples collected at the same time intervals after infusion of Advate. The prolonged hemostatic efficacy observed after rFVIIIFc infusion correlated well with the extended plasma circulation of rFVIIIFc, adding approximately 1 day to the 'time to trough' compared with Advate (Fig. 2).

Table 2 Mean rotation thromboelastometry clotting time ± SD

	Advate CT ± SD (s)	rFVIIIFc – Week 1 CT ± SD (s)	rFVIIIFc – Week 14 CT ± SD (s)
NATEM	730 ± 131	737 ± 173	721 ± 128
INTEM	495 ± 80	494 ± 73	497 ± 57
EXTEM	697 ± 187	754 ± 186	671 ± 96

Following infusion of rFVIII, rFVIIIFc (Week 1), and rFVIIIFc (Week 14). CT, clotting time; rFVIIIFc, recombinant factor VIII Fc.

Fig. 2

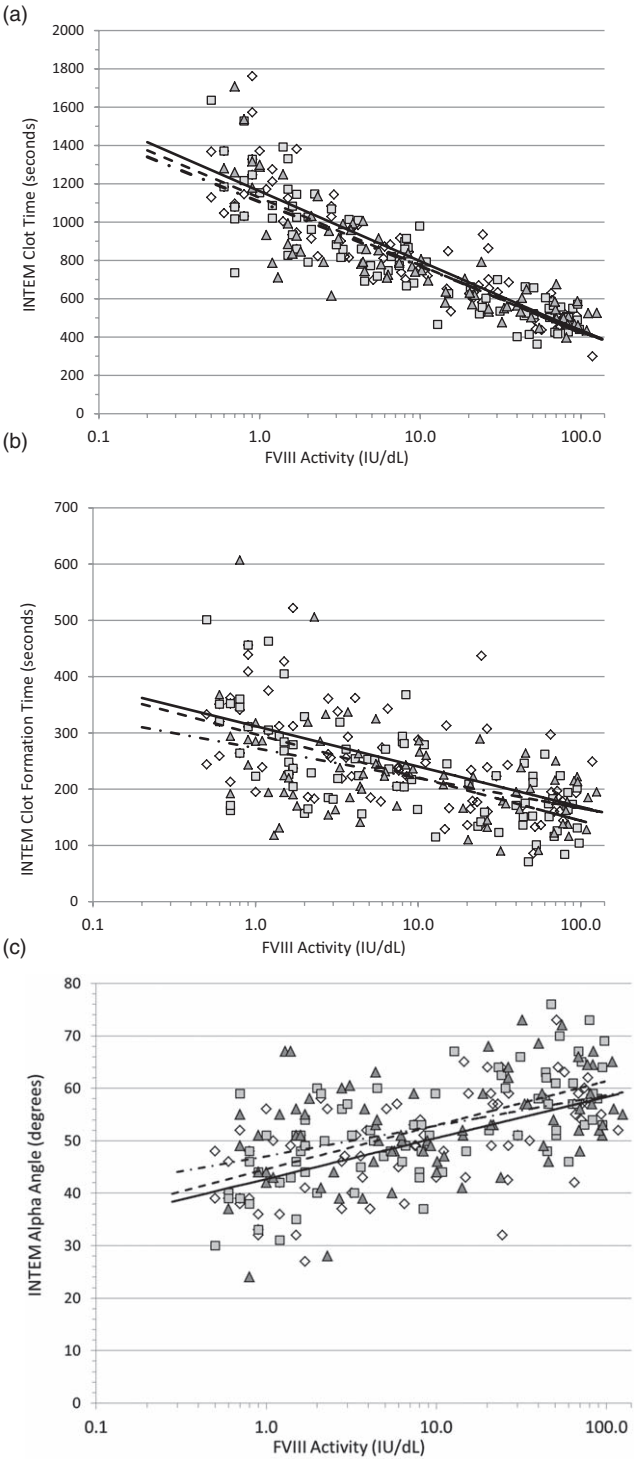


INTEM clot time vs. time profile shows that recombinant factor VIII Fc maintains whole blood clotting activity for a longer duration than Advate, corresponding to the higher plasma FVIII activity observed for recombinant factor VIII Fc between 1 and 5 days after infusion of the FVIII product. Symbols are mean results for 16 patients after infusion of 50-IU/kg Advate (diamonds), recombinant factor VIII Fc (Week 1, squares) and repeat recombinant factor VIII Fc administration (Week 14, triangles). Solid lines and symbols = FVIII activity by one stage clotting assay on a log scale; dotted lines and symbols = INTEM clot time on a linear scale.

Correlation of rotation thromboelastometry activity and one-stage clotting activity

The whole blood clotting activity varied considerably between the 16 individuals receiving the same dose of Advate/rFVIIIFc, as would be expected from a global hemostasis assay. A scatter plot of INTEM CT vs. FVIII activity for all samples with measurable FVIII more than 0.5 IU/ml by the one stage clotting assay is shown in Fig. 3a. Results from samples collected after the first rFVIIIFc pharmacokinetic evaluation (Week 1) were plotted separately from the 2nd (repeat) rFVIIIFc pharmacokinetic analysis (Week 14 ± 1). Log-linear regression analysis of these results demonstrated that the average ROTEM activity per FVIII unit for the 16 patients was reproducible between the two rFVIIIFc pharmacokinetic assessments. Furthermore, overlapping regression lines for Advate vs. rFVIIIFc samples indicate comparable hemostatic clotting potential for these two products at equivalent FVIII activity levels throughout the measurable assay range. Sample analysis by NATEM and INTEM confirmed equivalent hemostatic activity for rFVIIIFc and Advate per unit of FVIII activity by native or extrinsic activation of coagulation (data not shown). Intrapatient reproducibility between the first and second pharmacokinetic assessment was somewhat higher by the INTEM assay than by NATEM or EXTEM and further analysis was thus performed primarily based on the intrinsic activation of coagulation, in agreement with the recommendations by the Scientific

Fig. 3



Whole blood rotation thromboelastometry activity vs. plasma FVIII activity shows parallel, dose-dependent clotting time, clot formation time and alpha angle for Advate and recombinant factor VIII Fc, with slightly improved clot formation time, alpha angle and clot firmness in recombinant factor VIII Fc patients. The INTEM clot time (a), clot formation time (b), alpha angle (c). Open diamonds, solid line = Advate ($n = 77$); light-shaded squares, dashed line = recombinant factor VIII Fc (Week 1, $n = 81$); dark-shaded triangles, dash-dotted line = recombinant factor VIII Fc (Week 14, $n = 77$).

and Standardization Committee of the International Society of Thrombosis and Haemostasis [25].

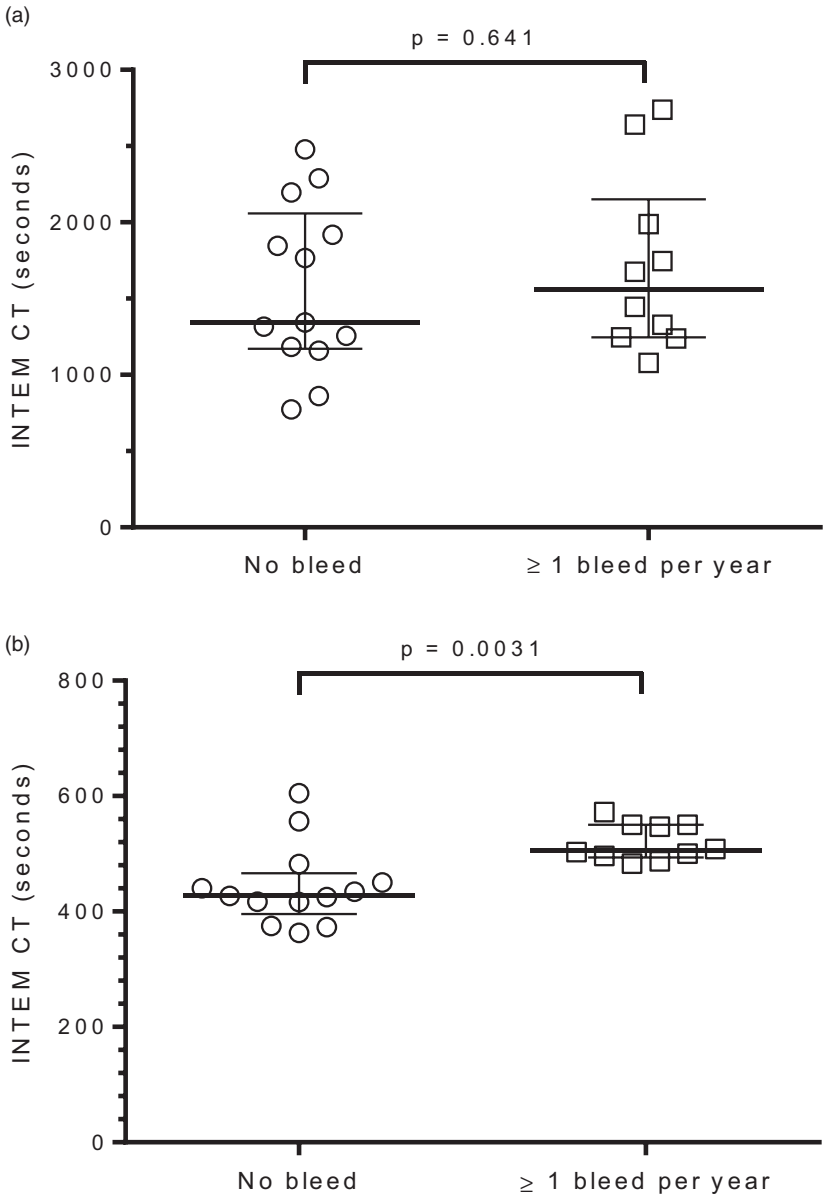
ROTEM CT represents the time to onset of clot formation after recalcification (clot initiation and amplification phase). We also evaluated clot formation time (INTEM CFT) and alpha angle (INTEM α) to compare the subsequent rate of clot formation (propagation phase) between rFVIIIFc and Advate. Figure 3b and c indicates slightly shorter CFT and steeper α angle on average for rFVIIIFc compared with Advate, but it is not clear whether these differences are significant. The MCF did not show a dependency on the level of FVIII activity. At very low or absent FVIII activity, some samples did not form a clot and therefore did not produce an MCF result during the run. For samples that did reach a maximal clot firmness at any time during the run, similar MCF results were observed for Advate and rFVIIIFc (data not shown).

Correlation of rotation thromboelastometry activity and bleeding incidence

The incidence of spontaneous breakthrough bleeds was monitored for 23 ROTEM patients that were on stable prophylaxis while enrolled in the A-LONG study and after rollover into the extension study for a total duration of 1 year. Patients in the ROTEM study that did not roll over into the extension study (did not have 1 year combined follow-up), or who did not remain on a stable regimen were excluded from this analysis as dose adjustments midyear due to bleeding events (or lack of bleeds) would have confounded the analysis. INTEM CT, CFT and α angle were evaluated for a possible correlation with the patient's bleed rate, including preinfusion and postinfusion values and estimated ROTEM values at trough during prophylaxis. Whole blood clotting activity after complete washout of replacement factor should represent the residual coagulation potential in the absence of any measurable FVIII and might thus be expected to correlate with a patient's bleeding tendency and clinical phenotype [11]. In our study, no correlation was observed between preinfusion ROTEM results and the occurrence of spontaneous bleeds over the 1-year observation period in these 23 patients (Fig. 4a). An association was however observed between postinfusion CT at the time of pharmacokinetic assessment and the occurrence of one or more spontaneous bleeds vs. no bleeds over a period of 1 year ($P = 0.0031$ by Mann–Whitney test, Fig. 4b). The median CT after infusion of 50 IU/kg rFVIIIFc for patients with at least one bleed ($n = 10$) was 506 s (range 483–572 s), whereas those patients without any bleeds ($n = 13$) had a median postinfusion CT of 427 s (range 363–605 s). The majority of the bleed-free patients (11/13) had a postinfusion CT below the lowest CT in the group with at least 1 bleed. The median FVIII activity at the time of postinfusion ROTEM measurement was 77.5 IU/dl in the bleed-free patients and 80.4 IU/dl in those with one or more spontaneous bleeds. No correlation was found between postinfusion FVIII levels and the occurrence of spontaneous bleeds ($P = 0.418$).

Maintaining a minimal trough level of FVIII activity is important for the prevention of spontaneous bleeding. We thus evaluated whether lower FVIII trough levels could correlate with the bleeding frequency in our patients. The median-estimated FVIII levels at trough based on each patient's pharmacokinetic parameters and dosing regimen were 2.10% (range 0.14–6.10%) in the bleed-free group and 1.85% (range 0.30–7.45%) in the patients with one or more bleeds. No significant difference was observed in the estimated trough FVIII activities between the two groups ($P = 0.250$). As the actual ROTEM measurements at predose (baseline) may have been unreliable due to high assay variability at very low FVIII levels, we estimated each patient's CT at trough by extrapolation from their more robust ROTEM data obtained at higher FVIII levels during the pharmacokinetic assessment. For each patient, the predicted CT was adjusted for his dosage and longest regular dosing interval. Lower interpatient variability was indeed observed for the estimated CTs at trough compared with the actual CTs measured at baseline (Fig. 5), but the difference between the bleeding vs. bleed-free group was again not statistically significant ($P = 0.238$). The 'time to 1% FVIII' is often derived from a patient's pharmacokinetic data to determine treatment intervals as prolonged time spent below 1 IU/dl FVIII increases the probability of spontaneous bleeds. Yet many patients require higher levels to avoid breakthrough bleeds whereas others exhibit no bleeds at trough levels below 1 IU/dl [26]. We chose a CT of 1000 s as an arbitrary 'target level' based on the observation that the median-estimated CT at trough for the 13 bleed-free patients was 1015 s. Extrapolating from the ROTEM measurements obtained at higher FVIII levels we predicted the 'time to 1000 s CT'. A good correlation was observed between 'time to 1000 s CT' and 'time to 1% FVIII' for 36 patients that had successfully completed both pharmacokinetic and ROTEM assessments ($R^2 = 0.70$, Fig. 6) which was expected as both parameters are a reflection of the patient's rate of FVIII clearance. Based on the patient's dosing intervals, we also estimated the 'time spent above 1000 s CT' for the 23 patients on stable prophylaxis, which may represent a period of inadequate factor coverage analogous to 'time spent under 1% FVIII'. On average, patients experiencing no bleeds during the 1-year observation period indeed spent less time above 1000 s CT than those with bleeds, with a difference in the median time of 0.43 days (Fig. 7). However, considerable variability was observed within each group, ranging from −1.5 to +2.5 days and the shorter time spent above 1000 s CT for bleed-free patients was not statistically significant ($P = 0.442$).

Fig. 4

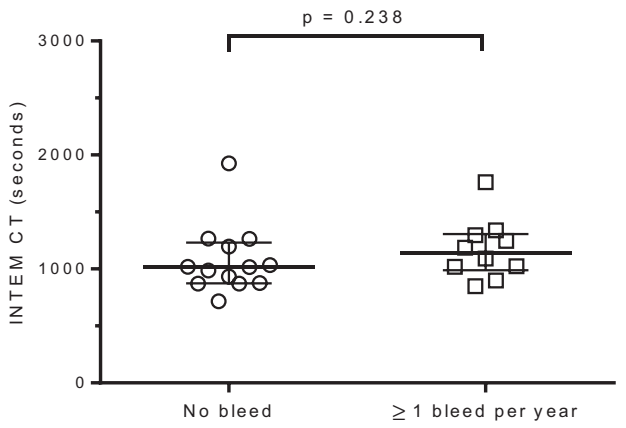


Preinfusion INTEM clotting time in 23 patients shows no difference between patients with or without spontaneous bleeds while on study for 1 year (a). Postinfusion INTEM clotting time 6 h after a dose of 50 IU/kg of recombinant factor VIII Fc is significantly shorter in patients without any bleeds (b). Open circles represent 13 patients that did not experience any spontaneous bleeding over a 1-year follow-up period. Open squares represent 10 patients with 1–9 bleeds per year. Bars = median with interquartile range.

Discussion

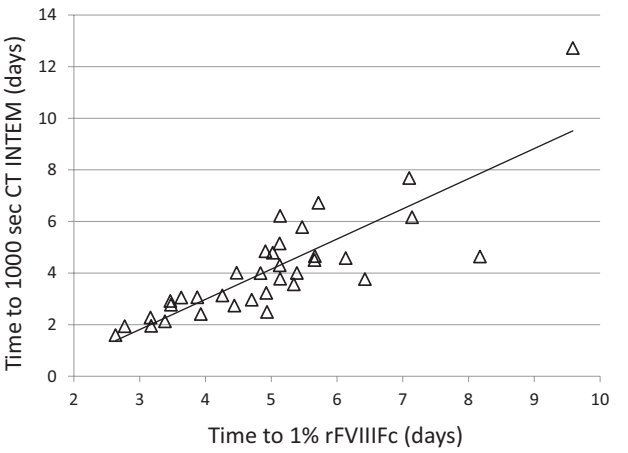
The choice of potency assay for a modified coagulation factor is critical if it is to provide the same in-vivo hemostatic efficacy per international unit (IU) as observed for conventional FVIII products. Although the efficacy of a FVIII product is formally determined by the clinical response during the treatment and prevention of bleeds, methods for quantifying the number of bleeds, or the number of doses required to treat a bleed, lack precision. Evaluating whole blood clotting activity by ROTEM or TEG is perhaps the closest surrogate method available for quantifying a patient's coagulation potential in response to a replacement factor. By comparing the relative performance of two products head to head in the same patient, interpatient and interlaboratory variabilities of ROTEM responses become essentially irrelevant. Our analysis of 16 patients undergoing sequential pharmacokinetic assessments included more than 140 rFVIIIFc, and 60 Advate samples for which both measurable plasma FVIII activity and ROTEM results

Fig. 5



Estimated INTEM clotting time at trough shows no difference in patients with or without any spontaneous bleeds on study. Clotting time s at trough were estimated based on a patient's dose and treatment intervals, and extrapolating from his INTEM clotting times measured at the time of pharmacokinetic assessment, assuming a linear increase of clotting time over time. Bars = median with interquartile range.

Fig. 6



Estimated time to 1000 s INTEM clotting time correlates with time to 1% FVIII activity after infusion of recombinant factor VIII Fc. The time to an INTEM clotting time of 1000 s and the time to 1% FVIII were extrapolated from the rotation thromboelastometry and FVIII activity measurements taken at the time of pharmacokinetic assessment following a dose of 50-IU/kg recombinant factor VIII Fc in 36 patients. ($R^2 = 0.699$).

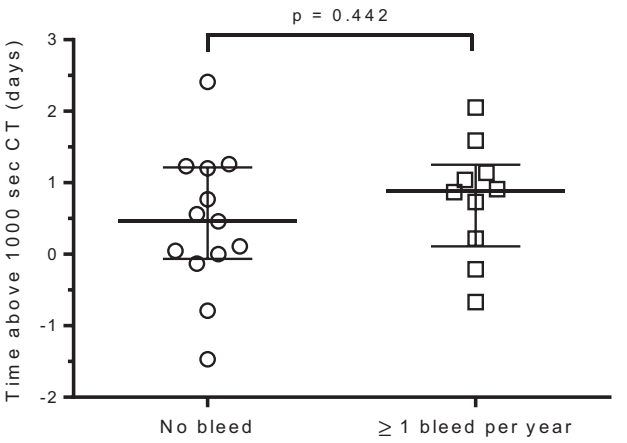
were available. On average, the INTEM whole blood CTs in postinfusion samples collected 6 h after administration of 50 IU/kg Advate or rFVIIIFc differed by less than 1%. Average INTEM CT results from the repeat analysis of postinfusion samples during the second rFVIIIFc pharmacokinetic assessment 3 months later also remained within 3% of the original values, confirming the good precision that could be achieved for this method by rigorous instrument and assay standardization.

Our results demonstrate that the potency assignment of rFVIIIFc provides a hemostatic efficacy per unit of FVIII that is indistinguishable from that observed for Advate, a well established full-length recombinant FVIII product. Equivalent in-vitro efficacy was also observed at low FVIII levels in which the average ROTEM CT per IU remained comparable between rFVIIIFc and Advate, as shown by the overlapping and parallel regression lines. The overall correlation between INTEM CT and measurable FVIII activity by the one-stage clotting assay was 0.761 (Pearson R^2) for 157 samples. We thus expect that rFVIIIFc provides hemostatic efficacy comparable with that of Advate throughout the entire measurable range of FVIII activity. Phlebotomy procedures likely varied between sites and in our experience even minor preactivation of samples can significantly shorten the CT in global hemostasis assays. In one of our own observations during this study, prolonged application of a tourniquet before and during blood collection resulted in a clot time of less than 400 s at baseline in the absence of any measurable FVIII (unpublished). Although a small number of such obvious outliers could be identified in our study and excluded from the analysis, significant impact of preanalytical variability on the baseline ROTEM data likely remained.

Comparable whole blood clotting potential for rFVIIIFc and Advate at equivalent doses was also demonstrated by EXTEM and by NATEM. However, INTEM CT using a 300-fold dilution of the vendor provided intrinsic activator (ellagic acid) appeared to be the most reliable method in our studies, typically demonstrating the lowest interassay variability in human samples, as well as in the plasma controls. Other studies also demonstrated that intrinsic activation provides more robust measurements than extrinsic TF-based activation of coagulation and that CT was less affected by interdevice variability than other ROTEM parameters [25,27]. In our study, α angle and CFT were also proportional to FVIII across the entire range, but significantly more scatter was observed for these parameters (Fig. 3b and c). Comparable MCF results were also achieved for rFVIIIFc and Advate, suggesting that the Fc modification in rFVIIIFc does not interfere in the later stages of clot formation or clot stability. Overall, our results suggested that equivalent efficacy may be achieved for rFVIIIFc and Advate in the treatment of acute bleeds and in maintaining hemostasis at trough levels. The overall safety and efficacy of rFVIIIFc was subsequently confirmed by our clinical studies [21].

Establishing a clear correlation between global hemostasis and a patient's bleeding phenotype has been an elusive goal, yet continues to be of interest in the management of hemophilia [8,10]. Intrapatient biological variability in coagulation factors over longer periods of

Fig. 7



Estimated time spent above 1000 s INTEM clotting time is not different between patients with or without any spontaneous bleed on study. A patient's time spent above 1000 s clotting time may represent a period of inadequate factor coverage analogous to 'time spent under 1% FVIII'. Bars = median with interquartile range.

time [5–7] may limit our ability to connect global hemostasis measurements with bleeding events that occur at later times. Temporal biological variability, combined with assay imprecision, may also be a reason why interpatient variation in global hemostasis measurements could thus far not be traced to persistent differences in particular clotting factors. Potentially, mathematical modeling of the coagulation process could more accurately predicted the limiting factor(s), with the finding by [28] that normal variation in TF pathway inhibitor may have the highest impact on the level of thrombin generation.

Relatively few spontaneous bleeds were observed in the 44 ROTEM study patients, and many patients adjusted their dosing regimen when observing breakthrough bleeds or in the prolonged absence of any bleeds. Despite these limitations, 23 patients were identified for which bleeding data were recorded over the course of a full year while on a stable prophylaxis regimen. In these patients, a higher postinfusion ROTEM activity (shorter clot times) was associated with the absence of spontaneous bleeding. Specifically, 11 patients who achieved an INTEM CT of 482 s or less in our test system following a 50-IU/kg dose of rFVIIIFc were bleed-free during the entire following year, whereas all patients with more than 482 CT ($n = 10$) experienced one or more bleeds. The extent to which a dose of FVIII corrects the clotting defect in a patient depends on the composition of his other procoagulant and anticoagulant factors [16,29]. We speculate that factors contributing to shorter clot times observed after infusion are also likely to influence residual hemostasis at very low levels of FVIII and may thus explain our observed correlation with bleeding tendency. Our data thus suggest that how well a patient's clotting deficiency is corrected after a dose of FVIII may be an indicator of subsequent bleeding tendency in patients with otherwise equivalent FVIII trough levels. Meanwhile, it is possible that small amounts of endogenous FVIII below 1% influence a patient's bleeding tendency during extended dosing intervals. Minor amounts of endogenous FVIII activity would, however, not contribute to the postinfusion ROTEM response. As it is difficult to accurately measure residual FVIII levels below 1%, an analysis of the patients FVIII genotype could be used to verify absence of residual endogenous activity. In a prospective study, limiting the analysis to those patients with an intron *22* inversion and other nonsense or null mutations may avoid the confounding effect of potential minor amounts of residual FVIII activity. In our study population, the genotypes were approximately evenly distributed between the two groups. The 13 bleed-free patients included five intron *22* inversions, three nonsense mutations and five other mutations (duplications, frameshift, splice or missense mutations). The 10 patients with one or more bleeds included four intron *22* inversions, one nonsense and five other mutations. Although the numbers were small, there appears to be no bias in the FVIII genotypes among the two groups. A postinfusion ROTEM or TEG measurement in patients without any measurable FVIII activity, perhaps in combination with a pharmacokinetic assessment, may provide an optimized dosing regimen for a given replacement factor, or a method for comparability with a modified clotting factor product in which plasma activity assays show discrepant results. If properly implemented, this approach might also result in lower factor consumption while minimizing breakthrough bleeds.

As there is considerable lot-to-lot variation in ROTEM/TEG reagents, a universal threshold value for a favorable whole blood CT in response to FVIII cannot be established on the basis of our results without common calibration standards. The consistency observed for our plasma control results suggest that normalizing local ROTEM data against frozen or lyophilized reference plasmas might reduce interlaboratory and lot-to-lot variation. In our study, the association of postinfusion CT with bleeding status was maintained, but not improved, when normalizing the ROTEM data against each site's average control plasma results at the 30% FVIII level (data not shown). Such normalization against a shared plasma control might nevertheless be effective when laboratories are using different reagents or assay procedures, as has been suggested previously for the thrombin generation test [30].

Conclusion

In conclusion, ex-vivo whole blood ROTEM analysis showed improved CT, CFT, and alpha angle for rFVIIIFc for a longer duration than Advate, consistent

with the higher FVIII activity by the one-stage and chromogenic assays reported previously. Furthermore, we demonstrated equivalent hemostatic activity per IU for rFVIIIFc and Advate, indicating that rFVIIIFc and Advate should be equally efficacious in maintaining hemostasis or in the treatment of bleeds. We also observed a striking association between faster postinfusion clot times by ROTEM and the absence of breakthrough bleeds in patients that had otherwise equivalent FVIII peak and trough levels. As our study was not designed or powered to establish correlations between bleeding phenotypes and global hemostasis measurements, larger prospective studies would be needed to validate the predictive value of a postinfusion whole blood clotting assay. The technical challenges of standardizing the commercial ROTEM assay, largely overcome in the current study, may however preclude the use of this method for widespread assessment of global hemostasis, unless additional assay controls or normalization procedures prove to be effective.

Acknowledgements

The following individuals contributed to the ROTEM studies: Anna Riddell (Royal Free Hospital, London, UK); Bernd Jilma (Medical University Vienna, Vienna, Austria); Grace Gilmore and Jim Thom (Royal Perth Hospital, Perth, Western Australia, Australia); Boris Shenkman (Sheba Medical Center, Tel Aviv, Israel); Colleen Engle (Bloodworks Northwest, Seattle, Washington, USA); Sean Wilkes and Latoya Lashley (Brigham and Women's Hospital, Boston, Massachusetts, USA); Tomoko Matsumoto, Kenichi Ogiwara and Koji Yada (Nara Medical University, Kashihara, Japan); Sean Platton (Barts Health NHS Trust, London, UK); Jane Needham (Basingstoke and North Hampshire Hospital, Basingstoke, Hampshire, UK); Cesar Guerrero (Children's Hospital Los Angeles, Los Angeles, California, USA); Stéphanie Francillon (Hôpital Édouard Herriot, Lyon, France), Elizabeth Donnachie (Gulf States Hemophilia & Thrombophilia Center, Huston, Texas, USA). Tem International GmbH (Munich, Germany) prepared custom diluents for this study.

Bioverativ and Sobi reviewed and provided feedback on the article. The authors had full editorial control of the article and provided their final approval of all content. This study was funded by Biogen and Bioverativ.

Conflicts of interest

M.G.M. was a research collaborator on this study and received no research funding or compensation for authorship for writing of this article. R.T.P. and J.M.S. were employees of Biogen at the time of the study and are now employees of Bioverativ. F.D. and G.F.P. were employees of Biogen during the time of the ROTEM study. F.D. is currently an employee of Novo Nordisk. G.F.P. is currently at the World Federation of Hemophilia.

References

1 Hartert H. Blutgerinnungsstudien mit der Thrombelastographie, einem neuen Untersuchungsverfahren. *Klinische Wochenschrift* 1948; **26**:37–38.
2 Luddington RJ. Thrombelastography/thromboelastometry. *Clin Lab Haematol* 2005; **27**:81–90.
3 Young G, Sørensen B, Dargaud Y, Negrier C, Brummel-Ziedins K, Key NS. Thrombin generation and whole blood viscoelastic assays in the management of hemophilia: current state of art and future perspectives. *Blood* 2013; **121**:1944–1950.
4 Furukawa S, Nogami K, Ogiwara K, Yada K, Minami H, Shima M. Systematic monitoring of hemostatic management in hemophilia A patients with inhibitor in the perioperative period using rotational thromboelastometry. *J Thromb Haemost* 2015; **13**:1279–1284.
5 Park KJ, Kwon EH, Ma Y, Park IA, Kim SW, Kim SH, *et al.* Significantly different coagulation factor activities underlying the variability of 'normal' activated partial thromboplastin time. *Blood Coagul Fibrinolysis* 2012; **23**:35–38.
6 Chen Q, Shou W, Wu W, Guo Y, Zhang Y, Huang C, *et al.* Biological and analytical variations of 16 parameters related to coagulation screening tests and the activity of coagulation factors. *Semin Thromb Hemost* 2015; **4**:336–341.
7 De Maat MP, van Schie M, Kluft C, Leebeek FW, Meijer P. Biological variation of hemostasis variables in thrombosis and bleeding: consequences for performance specifications. *Clin Chem* 2016; **62**:1639–1646.
8 Nogami K. The utility of thromboelastography in inherited and acquired bleeding disorders. *Br J Haematol* 2016; **174**:503–514.
9 Hoffman M, Monroe DM. A cell-based model of hemostasis. *Thromb Haemost* 2001; **85**:958–965.
10 Chitlur M, Young G. Global assays in hemophilia. *Semin Hematol* 2016; **53**:40–45.
11 Salinas V, Carmona R, Mohammed BM, Martin EJ, Brophy DF, Young G. Is some better than none: are TEG and TGA profiles different in severe FVIII-deficient patients with inhibitors? *Haemophilia* 2015; **21**: 398–404.
12 Al Hawaj MA, Martin EJ, Venitz J, Barrett JC, Kuhn JG, Nolte ME, *et al.* Monitoring rFVIII prophylaxis dosing using global haemostasis assays. *Haemophilia* 2013; **19**:409–414.
13 Antovic JP, Mikovic D, Elezovic I, Holmström M, Wilkens M, Elfvinge P, *et al.* Two global haemostatic assays as additional tools to monitor treatment in cases of haemophilia A. *Thromb Haemost* 2012; **108**:21–31.
14 Chitlur M, Warrier I, Rajpurkar M, Hollon W, Llanto L, Wiseman C, *et al.* Thromboelastography in children with coagulation factor deficiencies. *Br J Haematol* 2008; **142**:250–256.
15 Ghosh K, Shetty S, Kulkarni B. Correlation of thromboelastographic patterns with clinical presentation and rationale for use of antifibrinolytics in severe haemophilia patients. *Haemophilia* 2007; **13**:734–739.
16 Gissel M, Whelihan MF, Ferris LA, Mann KG, Rivard GE, Brummel-Ziedins KE. The influence of prophylactic factor VIII in severe haemophilia A. *Haemophilia* 2012; **18**:193–199.
17 Lewis SJ, Stephens E, Florou G, Macartney NJ, Hathaway LS, Knipping J, *et al.* Measurement of global haemostasis in severe haemophilia A following factor VIII infusion. *Br J Haematol* 2007; **138**:775–782.
18 Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Garagiola I, *et al.* Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. *J Thromb Haemost* 2010; **8**:737–743.
19 Rath T, Baker K, Dumont JA, Peters RT, Jiang H, Qiao SW, *et al.* Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. *Crit Rev Biotechnol* 2015; **35**:235–254.
20 Mahlangu J, Powell JS, Ragni MV, Chowdary P, Josephson NC, Pabinger I, *et al.* Phase 3 study of recombinant factor VIII Fc fusion protein in severe hemophilia A. *Blood* 2014; **123**:317–325.
21 Young G, Mahlangu J, Kulkarni R, Nolan B, Liesner R, Pasi J, *et al.* Recombinant factor VIII Fc fusion protein for the prevention and treatment of bleeding in children with severe hemophilia A. *J Thromb Haemost* 2015; **13**:967–977.
22 McCue J, Kshirsagar R, Selvitelli K, Lu Q, Zhang M, Mei B, *et al.* Manufacturing process used to produce long-acting recombinant factor VIII Fc fusion protein. *Biologicals* 2015; **43**:213–219.
23 Kitchen S, Kershaw G, Tiefenbacher S. Recombinant to modified factor VIII and factor IX – chromogenic and one-stage assays issues. *Haemophilia* 2016; **22 (Suppl 5)**:72–77.
24 Lippi G, Franchini M, Montagnana M, Salvagno GL, Poli G, Guidi GC. Quality and reliability of routine coagulation testing: can we trust that sample? *Blood Coagul Fibrinolysis* 2006; **17**:513–519.

25 Chitlur M, Rivard GE, Lillicrap D, Mann K, Shima M, Young G, Factor VIII, Factor IX, and Rare Coagulation Disorders Subcommittee of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. Recommendations for performing thromboelastography/thromboelastometry in hemophilia: communication from the SSC of the ISTH. *J Thromb Haemost* 2014; **12**:103–106.
26 Collins PW, Björkman S, Fischer K, Blanchette V, Oh M, Schroth P, *et al.* Factor VIII requirement to maintain a target plasma level in the prophylactic treatment of severe hemophilia A: influences of variance in pharmacokinetics and treatment. *J Thromb Haemost* 2010; **8**: 269–275.
27 Theusinger OM, Nürnberg J, Asmis LM, Seifert B, Spahn DR. Rotation thromboelastometry (ROTEM) stability and reproducibility over time. *Eur J Cardiothorac Surg* 2010; **37**:677–683.
28 Danforth CM, Orfeo T, Everse SJ, Mann KG, Brummel-Ziedins KE. Defining the boundaries of normal thrombin generation: investigations into hemostasis. *PLoS One* 2012; **7**:e30385.
29 Rendo P, Shafer F, Korth-Bradley JM, Sivamurthy K, Korin J. Factors that influence the bleeding phenotype in severe hemophilic patients. *Blood Coagul Fibrinolysis* 2013; **24**:683–690.
30 Dargaud Y, Luddington R, Gray E, Lecompte T, Siegemund T, Baglin T, *et al.* Standardisation of thrombin generation test – which reference plasma for TGT? An international multicentre study. *Thromb Res* 2010; **125**:353–356.