Thrombin generation to predict breakthrough bleeding in patients with acquired hemophilia A under emicizumab prophylaxis

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Abstract

Acquired hemophilia A (AHA) is a serious bleeding disorder due to neutralizing autoantibodies against factor VIII (FVIII). Emicizumab mimics the activity of FVIIIa restoring thrombin generation. It was shown to protect patients with AHA from bleeding, but some patients experience clinically relevant breakthrough bleeding. Therefore, monitoring the efficacy of emicizumab might be useful, potentially through thrombin generation assay (TGA). The aims of this study were to assess (i) how TGA is related to emicizumab levels, residual FVIII activity, and antigen concentration of other coagulation factors, and (ii) whether it can predict breakthrough bleeding during emicizumab prophylaxis. We used samples from patients enrolled in the GTH-AHA-EMI study that prospectively assessed the risk of bleeding in AHA patients receiving emicizumab for 12 weeks. Calibrated automated thrombogram assay was used with minute amounts of tissue factor (TF-TGA) or factor XIa (FXIa-TGA) to initiate coagulation. We observed that FXIa-TGA peak thrombin generation increased with emicizumab levels and FVIII activity. Higher peak thrombin values were associated with lower rates of bleeding as indicated by incident rate ratios (IRR) below 1 (IRR=0.40; 95% confidence interval: 0.17-0.84; P<0.05). TF-TGA was less sensitive to emicizumab and FVIII activity and was not associated with bleeding rate. FIX, FX and FXI antigen levels were not related to bleeding. In conclusion, FXIa-TGA was related to emicizumab levels and residual FVIII activity and to rates of clinically relevant bleeding. FXIa-TGA could be a useful biomarker to indicate increased risk of bleeding in patients with AHA emicizumab prophylaxis.

Introduction

Acquired hemophilia A (AHA) is caused by neutralizing autoantibodies against coagulation factor VIII (FVIII). Patients typically present with widespread subcutaneous bleeding but deep muscle hematomas, mucocutaneous, postoperative, or intracranial bleeding does also occur and can be life-threatening.[1,2] The disease is rare, but its reported incidence appears to have increased over time - from an estimated 1.48 cases per million per year in the UK in 2007[3] to 5-6 cases per million per year in Germany in 2021.[4] During the COVID-19 pandemic, reports of AHA associated with anti-SARS-CoV-2 vaccines increased over time, possibly reflecting the growing number of individuals vaccinated worldwide.[5] The traditional approach to AHA is to treat acute bleeding with bypassing agents (recombinant FVIIa or activated prothrombin complex concentrate), or with recombinant porcine FVIII (susoctocog alfa). To stop formation of FVIII-neutralizing autoantibodies, immunosuppressive therapy is applied.[1,2] The bispecific antibody emicizumab, that was developed for long-term prophylaxis in congenital hemophilia A,[6] has recently been considered to prevent bleeding in AHA patients until they achieve remission.[7-11]

The safety and efficacy of emicizumab in AHA is supported by two clinical trials,[12,13] case series,[14,15] and a real-world study from the US.[16] The GTH-AHA-EMI study demonstrated that rapid loading with emicizumab prevented bleeding in patients with AHA to a large degree, although study patients did not receive immunosuppression in the first 12 weeks and remained at low FVIII levels.[13] Propensity score-matched comparison to a historic cohort of patients treated with bypassing agents and immunosuppression demonstrated that GTH-AHA-EMI patients had significantly less bleeding.[17] Although this was considered a relevant improvement for patients with AHA, 14 of 47 patients (30%) still had between one and three breakthrough bleeds and half of them required hemostatic treatment. Baseline demographic and standard laboratory data were not related to the bleeding rate during 12 weeks of emicizumab prophylaxis.[18] Therefore, the search for better predictors of breakthrough bleeding in this situation is an important goal.

The objectives of the current study were (i) to determine how parameters of thrombin generation assays (TGA) are related to emicizumab levels, residual FVIII activity, and levels of other coagulation factors, and (ii) to find potential predictors of breakthrough bleeding while on emicizumab prophylaxis. We used stored samples from patients enrolled into the GTH-AHA-EMI clinical trial, since data and sample accrual of this study was prospectively planned providing the best possible quality for this analysis.

Methods

Study oversight

This study used samples collected from patients enrolled in the GTH-AHA-EMI trial (*clinicaltrials gov. Identifier: NCT04188639*). The objectives, eligibility criteria, and endpoints of this study have been published.[13] Patients were treated with emicizumab according to the study protocol with a loading dose of 6 mg per kilogram (kg) of body weight (day 1) and 3 mg/kg (day 2) subcutaneously, followed by a maintenance dose of 1.5 mg/kg per week from day 8 onwards until week 12. Samples for the current analysis were collected after emicizumab loading on visit (V)4 (day 8 to 10, N=26) or V5 (week 4, N=2). Detailed information on sample handling is provided in the *Online Supplementary Appendix*. Clinical data and outcomes were retrieved from the study database. All patients gave written informed consent before enrollment, including consent for biobanking. All study procedures were in accordance with the International Conference on Harmonization Guidelines for Good Clinical Practice and were approved by the regulatory authorities in Germany and Austria and by the ethics committees of all participating centers before initiation.

Laboratory assays

Thrombin generation was measured in platelet-poor plasma (PPP) using the Calibrated Automated Thrombogram (CAT, Stago, Asnières-sur-Seine, France). Peak thrombin generation (in nM), and endogenous thrombin potential (ETP, in nM/min) were used for analysis. Reference ranges were determined in age- and sex-matched controls. FVIII activity levels were measured using a chromogenic assay with bovine components to avoid interference of emicizumab. Coagulation factors IX, X, and XI were measured using immunoassays as the artificial shortening of the activated partial thromboplastin time (APTT) would falsely increase factor activity in standard activity assays. Detailed information on laboratory assays is provided in the *Online Supplementary Appendix*.

Statistical analysis

Data were summarized using appropriate descriptive statistics. Generalized linear modeling was applied to assess the effect of emicizumab and coagulation factor levels on TGA parameters (see *Online Supplementary Appendix*). Effect estimates were reported with the 95% confidence intervals (CI). Marginal effects of co-variates were plotted on the original scale against the response variable of interest. In addition, a simple nomogram was drawn using marginal effect sizes of the model. To analyze the association between the rate of clinically relevant bleeding (response variable, negative binomial distribution) and laboratory parameters, generalized linear models were used. Incident rate ratios (IRR) for bleeding were reported with 95% CI. Statistical significance was assumed for $P<0.05$. R version 4.3.0 and the 'tidyverse' package (2.0.0) were used for analysis and to prepare figures, with the 'MASS' package (7.3-60.0.1) for generalized linear models and the 'performance' package (0.15.0) to assess model performance.[19]

Results

Patient characteristics

Of the 47 patients enrolled in the GTH-AHA-EMI study, 28 patients had provided informed consent to biobanking and sufficient sample volume available for the current study (Figure 1). The baseline characteristics and the primary endpoint of this cohort are reported in Table 1. The age and sex distribution were similar to the original GTH-AHA-EMI study,[13] and representative of a typical AHA patient population. FVIII activity was markedly reduced to 2% of normal at baseline, and FVIII inhibitors were present in all patients at a median concentration of 14 BU/mL. Seventy-five percent of the patients had no breakthrough bleeding during the 12 weeks of emicizumab prophylaxis, while 25% had at least one bleeding event.

Thrombin generation assay characteristics

The semi-automated TGA used in this study had acceptable analytical precision. The day-to-day precision of control samples showed better coefficient of variation for FXIa-TGA (4-5%) compared to TF-TGA (8-15%; *Online Supplementary Figure S1*). Peak thrombin increased with emicizumab levels and FVIII activity over a broad range of levels with both assays, but FXIa-TGA was more sensitive to low concentrations of emicizumab or minute activities of FVIII (*Online Supplementary Figure S2*). Reference ranges derived from an age- and sex-matched control group are provided in *Online Supplementary Table S1*. A representative patient example of TF-TGA and FXIa-TGA before and after emicizumab loading is shown in *Online Supplementary Figure S3*.

Thrombin generation, emicizumab, and coagulation factor levels in patient samples

Samples for this analysis were drawn 1 week (V4, N=26) or 4 weeks (V5, N=2) after starting emicizumab. Emicizumab concentrations had reached steady state already 1 week after starting emicizumab.[13] The distribution of emicizumab levels, residual FVIII activity, antigen concentration of other coagulation factors, and TGA parameters are shown in Figure 2 and *Online Supplementary Table S2*.

Emicizumab levels measured by a modified FVIII activity one-stage assay were distributed around a median of 21 μg/mL (range, <10-49.2 μg/mL). Four patients had levels below the lower limit of quantification but shortening of the activated partial thromboplastin time still indicated that they had low levels of circulating emicizumab. FVIII activity levels - measured by chromogenic assay with bovine components to avoid interference of emicizumab - were reduced in all patients, and <5 IU/dL in most of them. Coagulation factors IX, X, and XI were within the reference range in most patients (Figure 2).

TGA parameters measured early after emicizumab loading indicated a partial correction of hemostatic function. In TF-TGA, both peak thrombin and ETP were below the

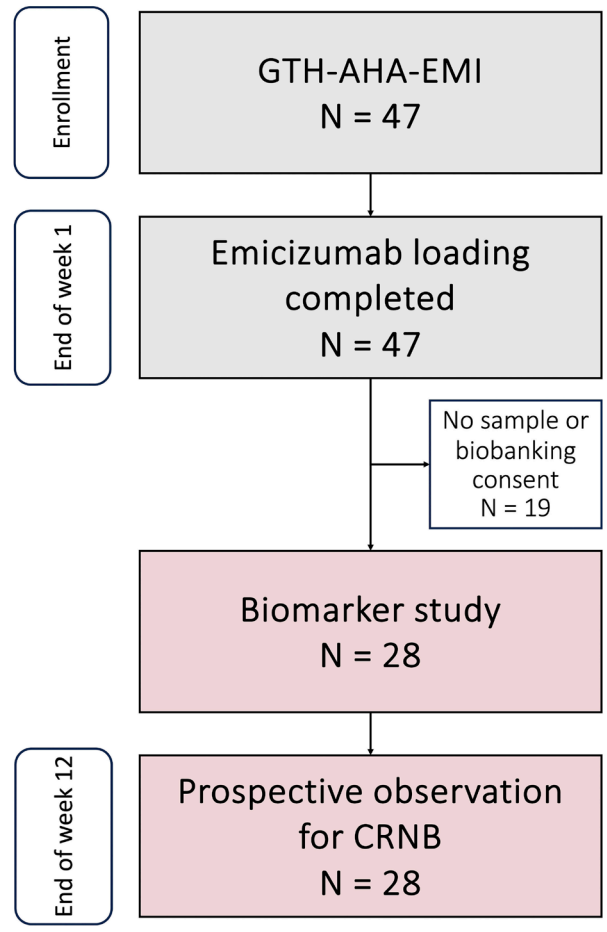

Figure 1. Patient disposition. Flow diagram of the study. Patients with newly diagnosed acquired hemophilia A (AHA), who were enrolled in the GTH-AHA-EMI clinical trial,[13] were eligible for the current biomarker study, if they had provided consent for biobanking and sufficient plasma volume available. The primary endpoint was the number of clinically relevant new bleeds (CRNB), counted from the first dose of emicizumab until the end of week 12.

Table 1. Baseline data and primary study endpoint.

Characteristic	All patients N=28
Baseline	
Age, years, median (IQR)	75 (66-79)
Sex, N (%)	
Female	12 (43)
Male	16 (57)
Underlying disorder, N (%)	
Malignancy	5 (18)
Autoimmunity	5 (18)
Postpartum	1 (3.6)
Baseline laboratory data	
Factor VIII activity, IU/dL, median (IQR)	2 (0-5)
Inhibitor, BU/mL, median (IQR)	14 (5-59)
Primary endpoint	
Clinically relevant bleeds during 12 weeks of emicizumab prophylaxis, N (%)	
0	21 (75)
1	3 (11)
2	4 (14)
3	0 (0)

IQR: interquartile range; BU: Bethesda units.

reference range in all patients. In FXIa-TGA, peak thrombin was variably reduced in most patients, while ETP values were within the normal range in the majority. This pattern mirrored findings from an *in vitro* spiking study (*Online Supplementary Figure S2*), which showed early saturation of FXIa-ETP in response to emicizumab and FVIII. Based on this observation, peak thrombin generation was selected as the primary parameter for further analysis in this study.

Thrombin generation according to levels of emicizumab and coagulation factors

The effect of emicizumab and coagulation factors on peak thrombin generation was assessed in patient samples for both TGA assays (*Online Supplement Table S3*). For the FXIa-TGA assay, peak thrombin generation significantly increased with both emicizumab levels and FVIII activity. For the TF-TGA assay, peak thrombin generation increased with emicizumab levels but not significantly with FVIII. Concentrations of FIX, FX, and FXI had no impact on either TGA assay. Multivariable analysis confirmed that emicizumab concentration and FVIII activity independently increased FXIa-TGA peak. This final model showed linearity over the range of emicizumab and FVIII levels in the study (*Online Supplementary Figure S4*).

Marginal effects of changes in emicizumab concentration (while holding FVIII activity at its average level) and changes in FVIII activity (while holding emicizumab concentration at its average level) on FXIa-TGA peak are illustrated in Figure 3. Univariate correlations between FXIa-TGA peak and FVIII, emicizumab, and the composite of FVIII and emicizumab are shown in *Online Supplementary Figure S5*. Based on the multivariable model, we constructed a simple nomogram that can be used to estimate FXIa-TGA peak from FVIII activity and emicizumab level (Figure 4).

Thrombin generation and bleeding rate

TGA parameters and coagulation factor levels were assessed in relation to the number of clinically relevant breakthrough bleeds during 12 weeks of emicizumab prophylaxis (Table 2). Higher FXIa-TGA peak values were associated with a

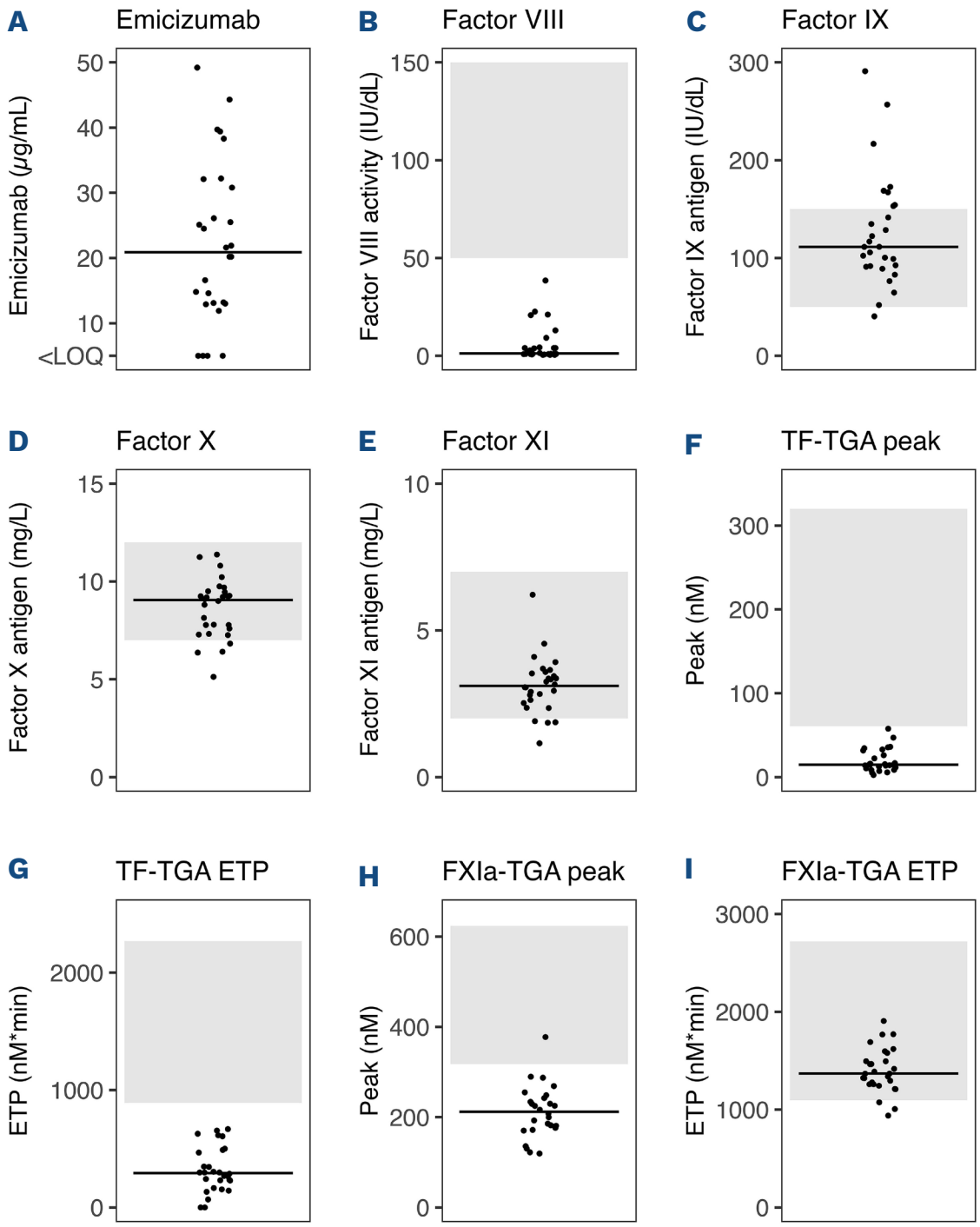

Figure 2. Distribution of laboratory data early after emicizumab loading. (A) Emicizumab level (by modified one-stage clot assay). (B) Factor VIII (FVIII) activity (chromogenic assay). (C) FIX (antigen assay). (D) FX (antigen assay). (E) FXI (antigen assay). (F) Tissue factor thrombin generation assay (TF-TGA) peak. (G) TF-TGA endogenous thrombin potential (ETP). (H) FXIa-TGA peak. (I) FXIa-TGA ETP. Dots indicate individual acquired hemophilia A (AHA) patients (N=28); horizontal lines indicate the median. Grey shaded areas indicate the reference interval. LOQ denotes the lower limit of quantification of emicizumab (<10 µg/mL).

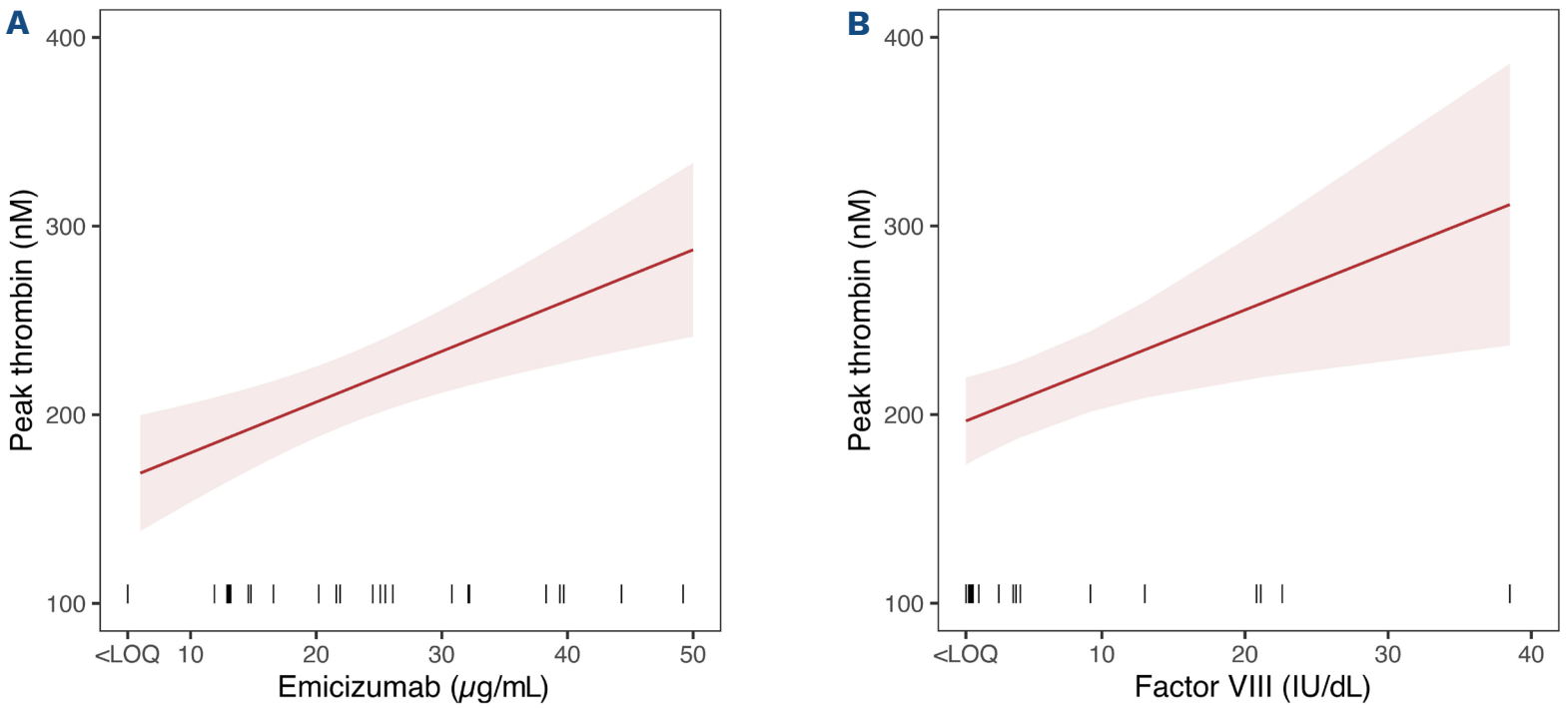

Figure 3. FXIa-thrombin generation assay peak according to emicizumab concentration and FVIII activity. Marginal effects of multivariable model of factor XI thrombin generation assay (FXI-TGA) peak according to (A) emicizumab level and (B) factor VIII (FVIII) activity early after emicizumab loading in 28 patients. Rug marks along the X axis indicate the distribution of emicizumab levels and FVIII activity in the data set.

lower rate of bleeding as indicated by an IRR significantly below 1. TF-TGA parameters, FVIII activity, inhibitor, and emicizumab levels were not associated with bleeding.

The bleeding rate declined with increasing FXIa-TGA peak values (Figure 5A). The association between FXIa-TGA parameters and risk of bleeding became also apparent when raw patient data were plotted (Figure 5B). The proportion of patients with bleeding was 4-fold higher, and the average number of bleeds 8-fold higher, when FXIa-TGA peak levels were ≤200 nM and ETP ≤1,400 nM/min (boxed area).

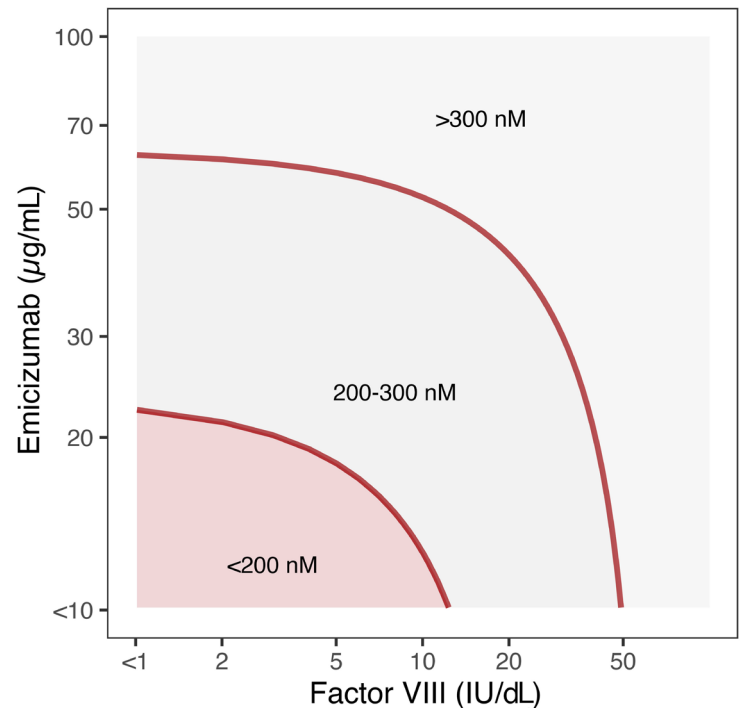

Figure 4. Nomogram to estimate FXIa-thrombin generation assay peak thrombin from FVIII activity and emicizumab. Data are derived from the model shown in *Online Supplementary Table S3*. Axes are logarithmic. The red curves mark factor XIa thrombin generation assay (FXIa-TGA) peak values of 200 and 300 nM.

Representative case studies

To illustrate the risk of bleeding according to FXIa-TGA peak, emicizumab levels, and FVIII activity, three representative case studies are presented (Figure 6).

Patient 1

A 37-year-old woman with secondary postpartum hemorrhage was diagnosed with AHA. After admission to the study site, she received recombinant porcine FVIII and started emicizumab prophylaxis. At the end of week 1, bleeding was under control, and she had an emicizumab level of 39.7 µg/ml. FVIII activity remained low at 0.4 IU/dL and the inhibitor was 4.3 BU/mL. FXIa-TGA peak (287 nM) was one of the highest within the study population, and she did not experience further bleeding.

Patient 2

A 70-year-old woman had multiple skin, muscle, and gastro-intestinal bleeding episodes before a diagnosis of AHA was made. After fast emicizumab loading, she had a low level of emicizumab (14.6 µg/mL), but her FVIII activity had already recovered to 22 IU/dL with the inhibitor declining to 1.1 BU/mL. FXIa-TGA peak (225 nM) was near the average of the population, and she did not suffer new bleeds during the study.

Patient 3

A 66-year-old woman was diagnosed with AHA due to multiple skin ecchymoses and gastro-intestinal bleeding. One week after starting emicizumab loading, her emicizumab level was only 13.1 µg/mL. FVIII activity was low at 4.3 IU/dL and the inhibitor 4.6 BU/mL. FXIa-TGA peak (119 nM) was in

the lower range of the cohort. Her bleeding tendency was not very well controlled as she experienced non-relevant urogenital bleeding weeks 2 and 3, and two episodes of clinically relevant urogenital and gastrointestinal bleeding in week 5.

Discussion

This study had the objective to describe how thrombin generation is related to emicizumab levels, residual FVIII activity and other coagulation factors in AHA. The second objective was to assess potential predictors of breakthrough bleeding. We found that FXIa-TGA peak levels were related to both emicizumab and FVIII activity, whereas coagulation factors IX, X, and XI did not significantly impact thrombin generation. FXIa-TGA early after emicizumab loading was related to the rate of breakthrough bleeding during the rest of the observation period. Tissue factor TGA (TF-TGA) was not clearly related to residual FVIII activity and did not predict bleeding.

These findings are consistent with a biologically plausible hypothesis. In AHA patients not treated with emicizumab, we previously reported that residual FVIII activity correlates with the rate of clinically relevant new bleeds over a 12-week observation period.[20] In patients receiving emicizumab, it is reasonable to assume that breakthrough bleeding risk would be influenced by both emicizumab concentration and residual FVIII activity. Our data suggest that FXIa-triggered thrombin generation integrates both variables, making it a plausible surrogate marker. Accordingly, the observed association between FXIa-TGA peak and bleeding rate supports this hypothesis.

The FXIa-TGA in our study was a modified in-house CAT assay that used low amounts of FXIa to trigger coagulation. Similar assays have recently been used in clinical studies with FVIII concentrates,[21] emicizumab,[22] and the preclinical development of other FVIIIa-mimetics.[23] These studies already suggested that FXIa-TGA is very sensitive to emicizumab and FVIII.

FXIa-TGA, but not TF-TGA, was associated with the risk of breakthrough bleeding in our patients. This difference likely reflects the mechanism of action of emicizumab.

Table 2. Clinically relevant bleeding according to laboratory markers.

Covariate	IRR (95% CI)
FXIa-TGA peak thrombin	0.40 (0.17-0.84)*
TF-TGA peak thrombin	1.39 (0.73-2.75)
Emicizumab	0.77 (0.32-1.68)
FVIII	0.54 (0.09-1.52)
Inhibitor	1.13 (0.47-2.68)

Incident rate ratios (IRR) from univariate generalized linear models with number of bleeds during 12 weeks of emicizumab (dependent variable, negative binomial distribution) according to z-transformed covariates. An IRR below 1 indicates a lower rate of bleeding. *P<0.05. CI: confidence interval; FXIa; factor Xia; TGA: thrombin generation assay; TF: tissue factor; FVIII: factor VIII.

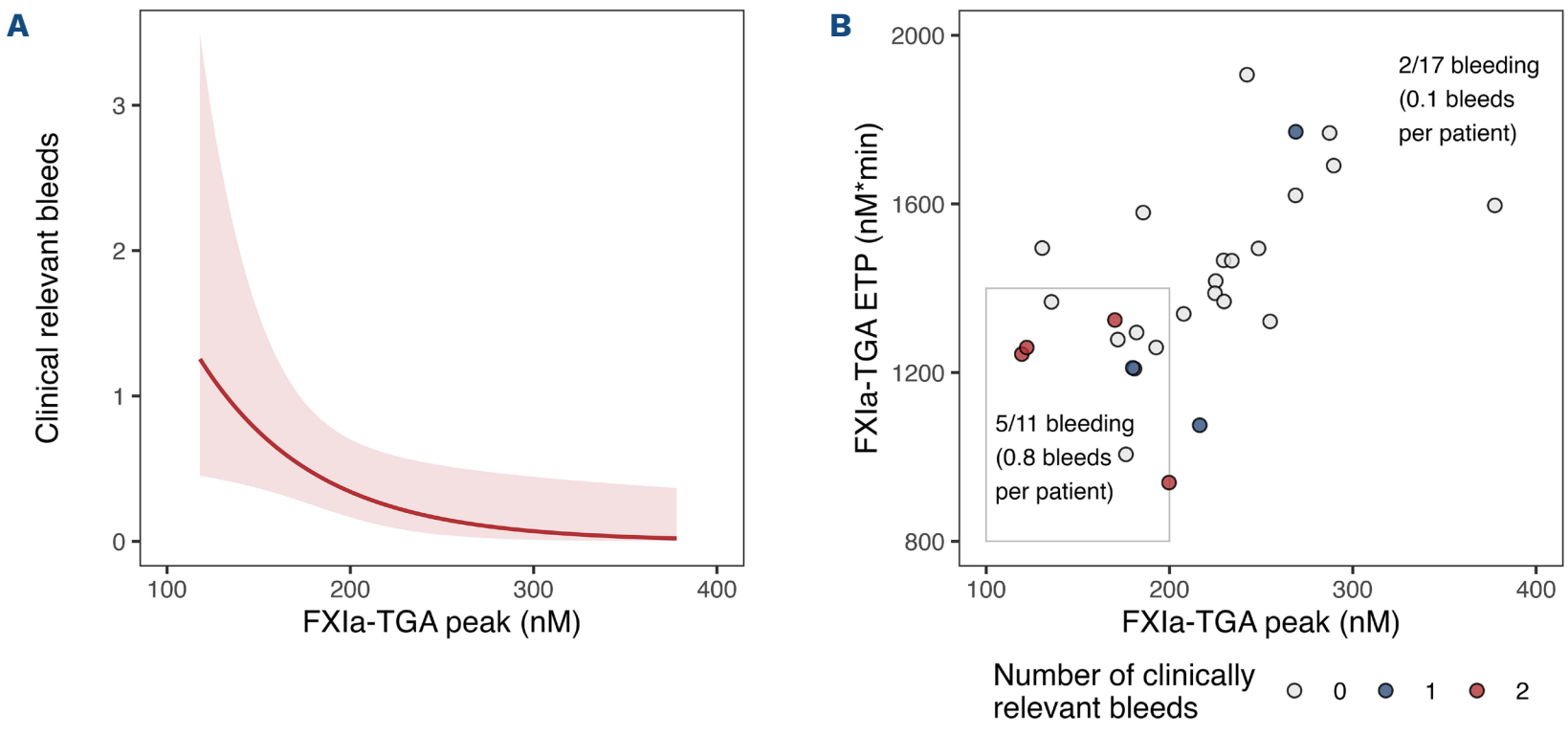

Figure 5. FXIa-thrombin generation assay peak thrombin and bleeding risk. (A) Mean number and 95% confidence interval (CI) of clinically relevant bleeds during 12 weeks of emicizumab according to factor XIa thrombin generation assay (FXIa-TGA) peak level early after emicizumab loading. The model was derived from data shown in Table 2. (B) Original patient data of FXIa-TGA peak and ETP early after emicizumab loading, with dot colors encoding the number of bleeding events during the subsequent period of emicizumab prophylaxis. The boxed area highlights cases with FXIa-TGA peak ≤200 nM and ETP ≤1,400 nM/min and denotes the proportion of bleeding patients and the mean rate of bleeds per patient.

Emicizumab facilitates FX activation by FIXa. FIX is more efficiently activated by FXIa than by the TF/FVIIa complex. K^D-based simulations predicted that emicizumab circulates partially bound to FIX via its EGF domain in plasma, with a small fraction forming a FIX-emicizumab-FX ternary complex.[24] This complex is believed to reflect the amount of FIX/FIXa-emicizumab-FX/FXa present on the phospholipid surface at a bleeding site. As in the FXIa-TGA assay,

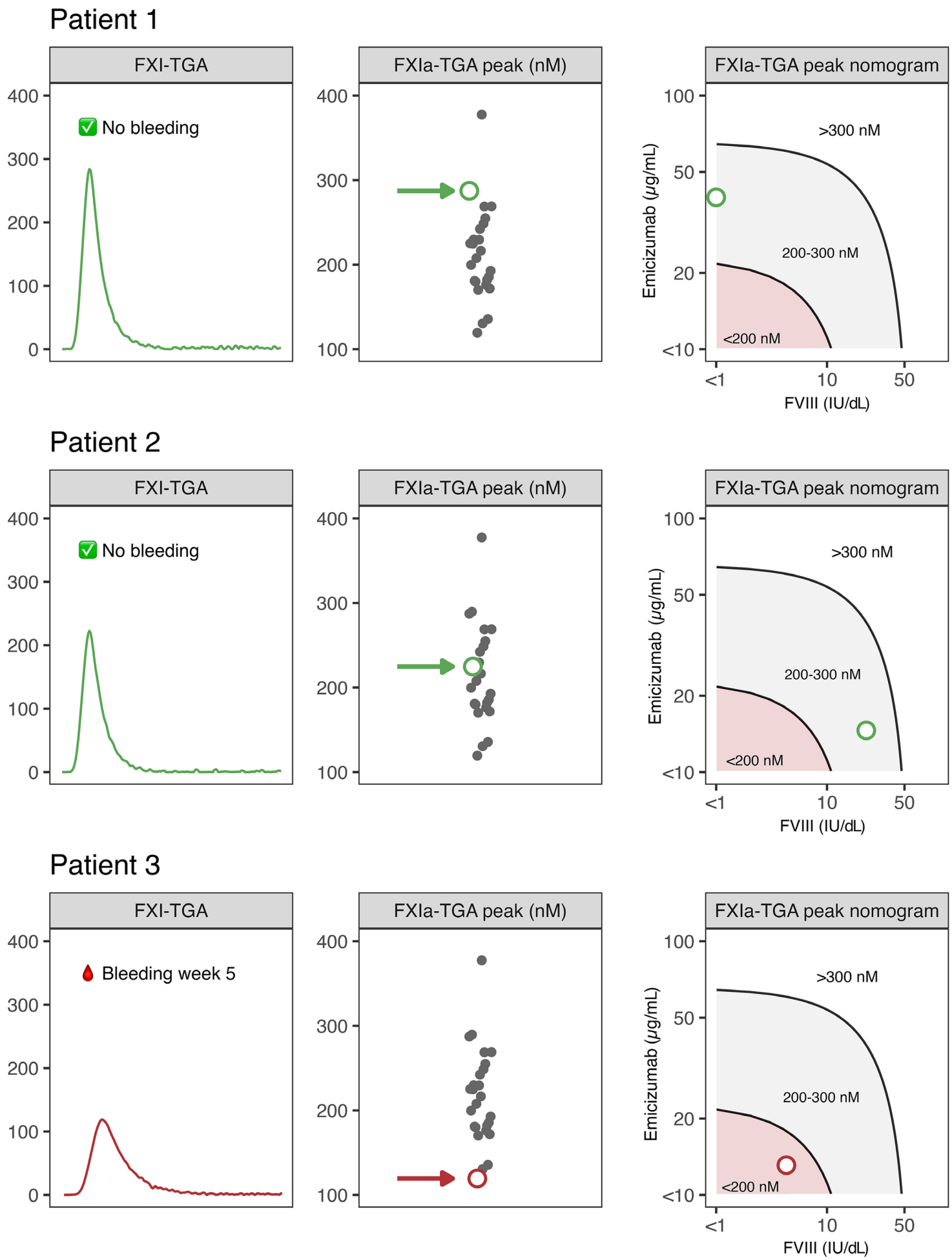

Figure 6. Representative examples. The panels show 3 individual patients described in the text. For each patient (arranged in rows), the factor XI thrombin generation assay (FXI-TGA) trace (left) with FXIa-TGA peak (middle), emicizumab concentration and FVIII activity are shown within the nomogram (right), highlighting their individual parameters (arrow) within the patient cohort. All data taken 1 week after emicizumab loading (visit 4).

the rate-limiting step in this physiological context is the activation of FIX, which may explain the closer relationship between FXIa-TGA results and emicizumab/FVIII concentrations.

The TF-TGA used in our study was the commercially available CAT assay. It employs ready-to-use reagents with minute amounts of TF and is considered state-of-the-art for assessing thrombin generation in bleeding disorders. Attempts have been made to standardize the assay and it has been used in many previous studies.[25] For example, it was used with success to personalize treatment of breakthrough bleeding with bypassing agents.[26] However, despite its correlation to residual factor activity in congenital hemophilia it failed to correlate with the bleeding score in non-inhibitor patients.[27] Attempts to predict bleeding rates or dosing requirements in congenital hemophilia patients were also unsuccessful.[28-30] The assay's inability to discriminate very low levels of FVIII could be a reason for this.[31] For novel hemophilia treatments like factor mimetics or re-balancing agents, TGA appears to be a logical candidate assay for pharmacodynamic drug monitoring.[32] A recent multicenter study reported that a subgroup of bleeding patients among a sizable cohort of severe congenital hemophilia A patients on emicizumab had lower thrombin generation when the assay was triggered with minute amounts of TF and FXIa together.[33] Dual TF/FXIa TGA was suggested to optimize the sensitivity and reproducibility of TGA to detect low amounts of FVIII in patients with severe congenital hemophilia A.[31] Our observation that FXIa-TGA was more closely related to FVIII activity and the bleeding rate of AHA patients on emicizumab is in line with these observations.

Data on thrombin generation in AHA are scarce. One study compared ten patients with AHA to 44 patients with lupus anticoagulant and concluded that the two conditions could be differentiated by TF-TGA peak levels.[34] Another study reported that TF-TGA parameters were severely impaired in AHA and improved when patients achieved remission.[35] An *in vitro* spiking study using plasma from AHA patients showed enhancement of TF-TGA after the addition of emicizumab.[36] However, reports correlating TF-TGA parameters with clinical bleeding severity, or the efficacy of hemostatic treatments, remained anecdotal.[37] Our study is the first prospective cohort study to suggest that TGA monitoring might be useful to predict the bleeding risk in AHA patients. Summarizing the recent findings from TF-TGA and FXIa-TGA studies in congenital hemophilia and AHA, including our own, it seems reasonable to further explore FXIa-TGA (rather than TF-TGA) as a predictor of the *in vivo* efficacy of FVIIIa mimetic drugs. This is particularly important as second-generation bispecific antibodies are being developed, which could enhance bleed protection in patients with AHA.

Our study has a few limitations. First, we could not enroll all GTH-AHA-EMI patients as not all participants provided consent for biobanking. Second, the number of bleeding events available for modeling was relatively small. Third, FXIa-TGA was an in-house assay that is not readily available for routine laboratories and lacks standardization. The effects of FVIII activity and emicizumab concentration on peak thrombin generation in the FXIa-TGA are highly dependent on the final concentration and activity of FXIa in the assay. Since FXIa is inactivated by antithrombin, even minor variations in assay conditions may influence its responsiveness to FVIII or emicizumab. This could affect both the precision of the nomogram we provide for estimating peak thrombin levels and the validity of the FXIa-TGA threshold of ≤200 nM, which was associated with increased bleeding risk in our study. Despite these limitations, we believe that our study offers valuable insight into monitoring hemostasis in AHA patients receiving emicizumab prophylaxis and identifying those at high risk of breakthrough bleeding.

In conclusion, FXIa-TGA was related to residual FVIII activity and emicizumab levels early after administration of loading doses. Low FXIa-TGA parameters were associated with clinically relevant bleeding events in our study. Our results encourage the conduct of larger observational studies establishing the use of FXIa-TGA to identify AHA at increased risk of breakthrough bleeding while on prophylaxis with emicizumab.

Disclosures

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Contributions

SW and AT conceived the study, guided the analysis, and wrote the manuscript. SW supervised laboratory analysis. FJP, EE, OO, and AK performed the assays and interpreted data. PK, CP, RG, JO, UJS, WM, KTG, KH, HE, PM, CH, RK, and AT enrolled patients and acquired samples. AT performed statistical analysis. All authors revised the manuscript and approved of its final version.

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Data-sharing statement

Original data will be made available by the corresponding authors upon reasonable request.

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