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

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Short title: Thrombin generation in AHA

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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 into 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 below 1 (IRR 0.40 [0.17-0.84], $p<0.05$). TF-TGA was less sensitive to emicizumab and FVIII activity and was not associated with bleeding rate. Factor IX, X and XI 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 factor VIIa 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 United States (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 1 and 3 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 (NCT04188639, registered at www.clinicaltrials.gov). 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 V4 (day 8 to 10, n=26) or V5 (week 4, n=2). Detailed information on sample handling is provided in the Supplement. 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 Harmonisation 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 Supplement.

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 Supplement for details). Effect estimates were reported with the 95% confidence intervals (CI). Marginal effects of covariates 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 forty-seven 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. 75% 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 semiautomated 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%, Supplement Figure 1). 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 (Supplement Figure 2). Reference ranges derived from an age- and sex-matched control group are provided in Supplement Table 1. A representative patient example of TF-TGA and FXIa-TGA before and after emicizumab loading is shown in Supplement Figure 3.

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

Samples for this analysis were drawn one week (V4, n=26) or four weeks (V5, n=2) after starting emicizumab. Emicizumab concentrations had reached steady state already one 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 Supplement Table 2.

Emicizumab levels measured by a modified FVIII activity one stage assay were distributed around a median of 21 µg/ml (range <10 to 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 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 (Supplementary Figure 2),

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 (Supplement Table 3). 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 (Supplement Figure 4).

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 Supplement Figure 5. 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 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 4fold higher, and the average number of bleeds 8fold higher, when FXIa-TGA peak levels were ≤200 nM and ETP ≤1400 nM*min (boxed area).

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 gastrointestinal 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 gastrointestinal 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. 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. 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, 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 rebalancing 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 10 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.

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Tables

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%)

Abbreviations: IQR, interquartile range

Table 2 Clinically relevant bleeding according to laboratory markers

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.

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)

* $p < 0.05$

Figures

Figure 1 Patient disposition

Flow diagram of the study. Patients with newly diagnosed AHA, who were enrolled in the GTH-AHA-EMI clinical trial (ref. 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.

Figure 2 Distribution of laboratory data early after emicizumab loading

(A) Emicizumab level (by modified one-stage clot assay). (B) FVIII activity (chromogenic assay). (C) Factor IX (antigen assay). (D) Factor X (antigen assay). (E) Factor XI (antigen assay). (F) TF-TGA peak. (G) TF-TGA ETP. (H) FXIa-TGA peak. (I) FXIa-TGA ETP. Dots indicate individual 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-TGA peak according to emicizumab concentration and FVIII activity

Marginal effects of multivariable model of FXI-TGA peak according to (**A**) emicizumab level and (**B**) 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.

Figure 4 Nomogram to estimate FXIa-TGA peak thrombin from FVIII activity and emicizumab

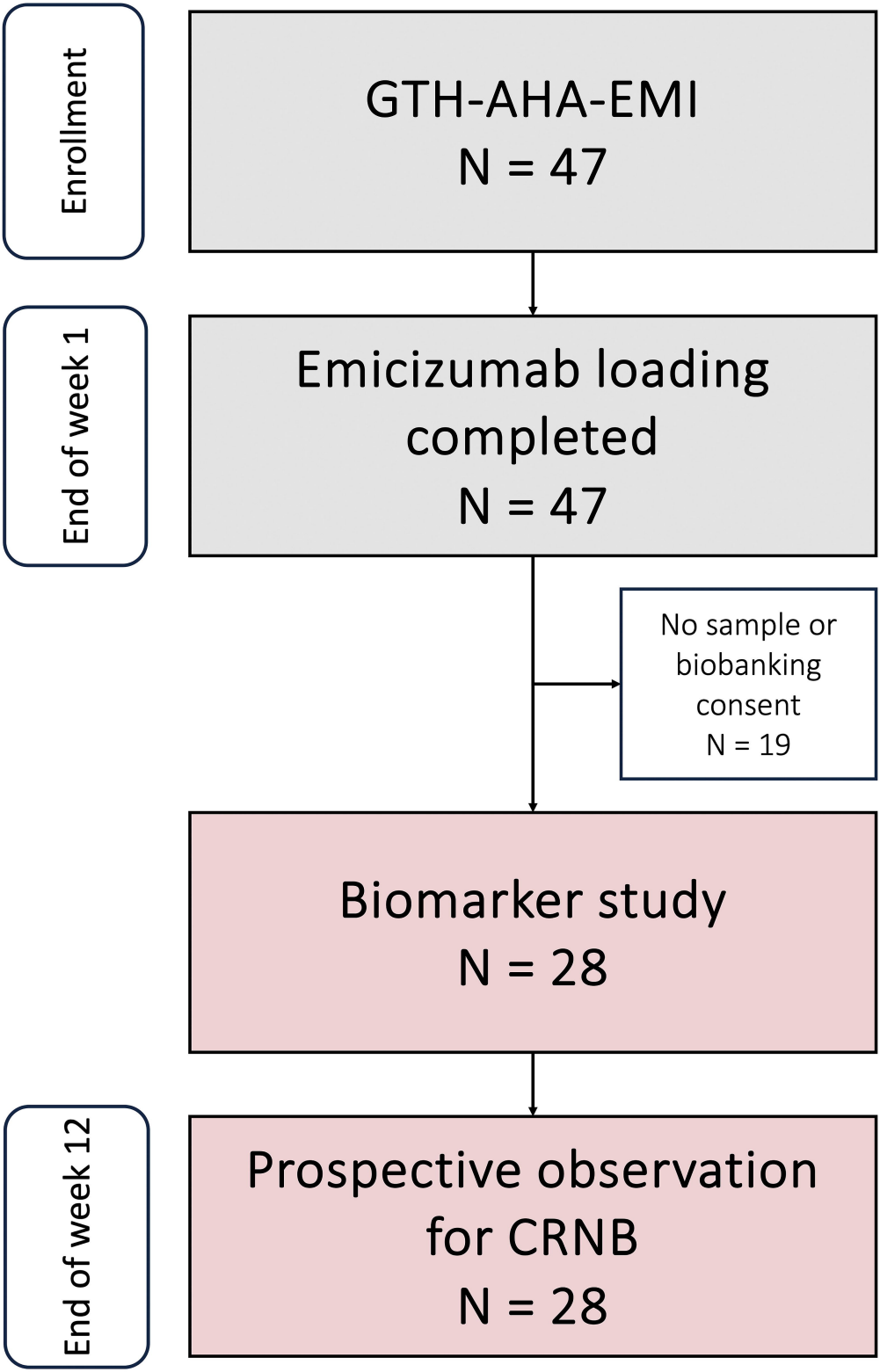
Data are derived from the model in Supplement Table 3. Axes are logarithmic. The red curves mark FXIa-TGA peak values of 200 and 300 nM.

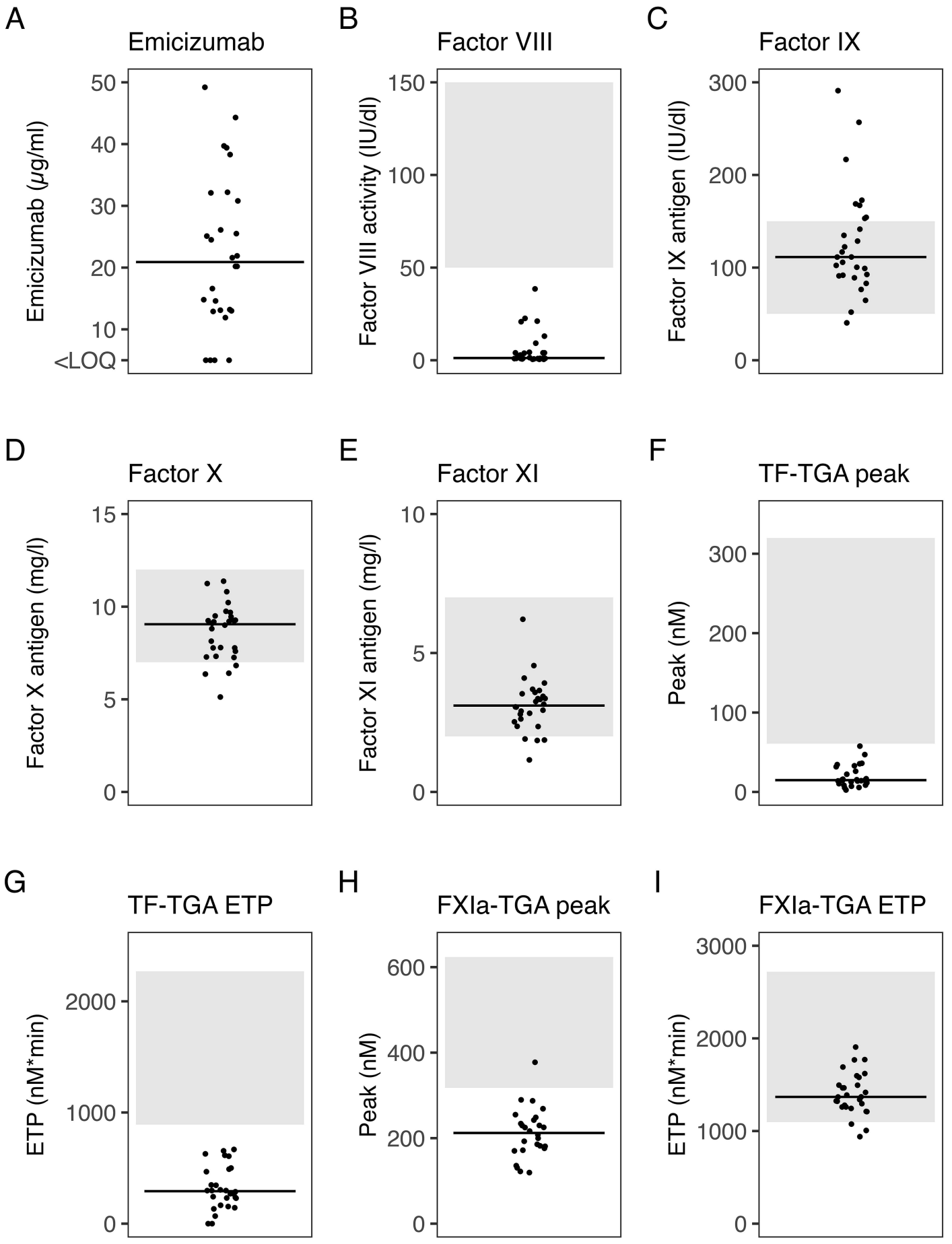
Figure 5 FXIa-TGA peak thrombin and bleeding risk

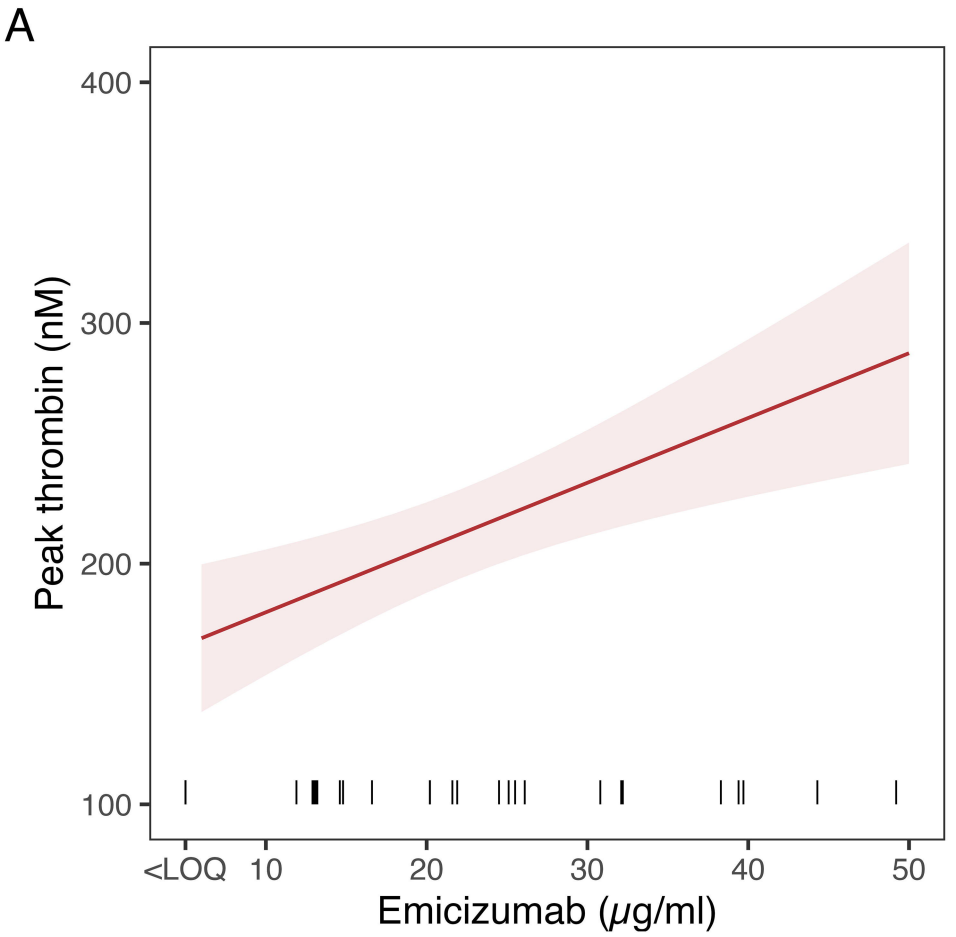
(A) Mean number and 95% CI of clinically relevant bleeds during 12 weeks of emicizumab according to FXIa-TGA peak level early after emicizumab loading. The model was derived from data in Table 2. (B) Original patient data of FXIa-TGA peak and ETP early after emicizumab loading, with dot colors encoding 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 ≤1400 nM*min and denotes the proportion of bleeding patients and the mean rate of bleeds per patient.

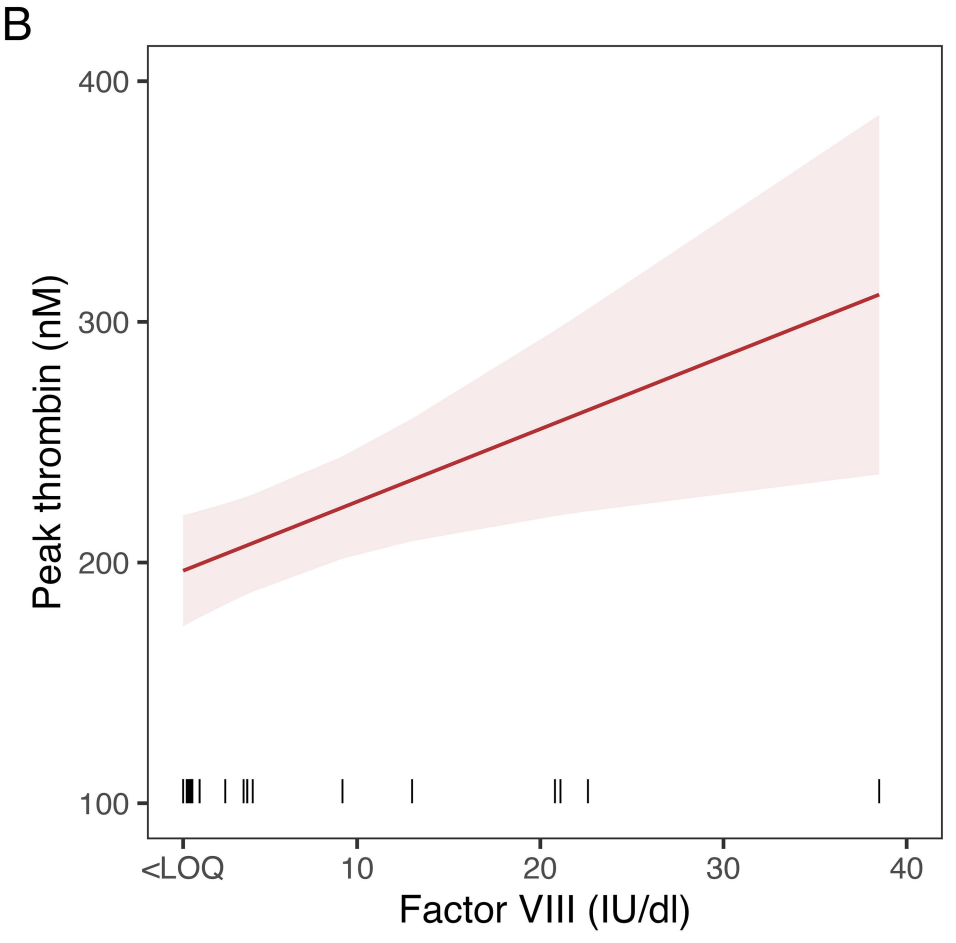
Figure 6 Representative examples

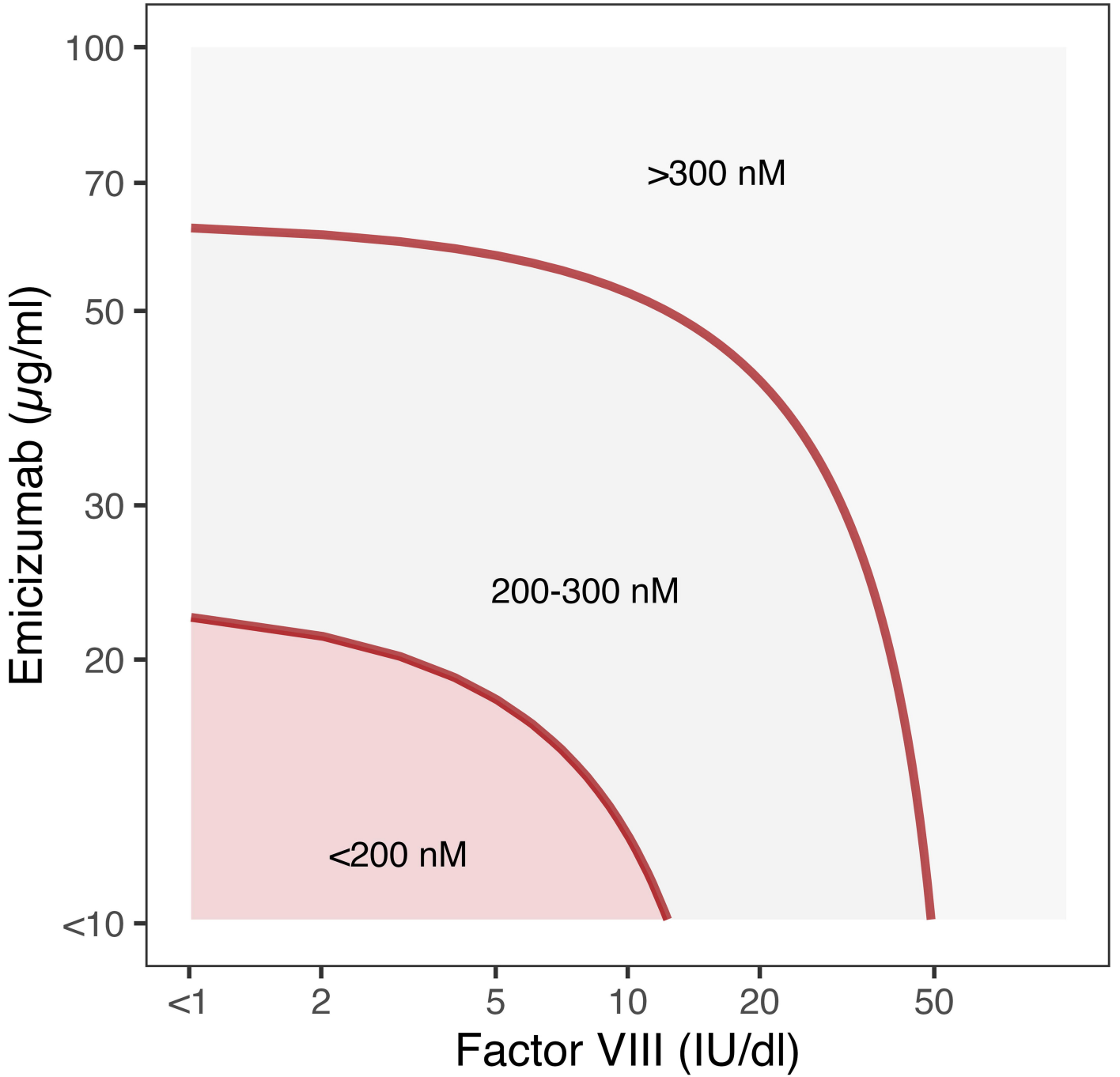
The panels show three individual patients described in the text. For each patient (arranged in rows), the FXI-TGA trace (left) with FXIa-TGA peak (middle), emicizumab concentration and FVIII activity are shown within the nomogram (right), highlighting their individual parameters (arrow) within the patient cohort. All data taken one week after emicizumab loading (visit 4).

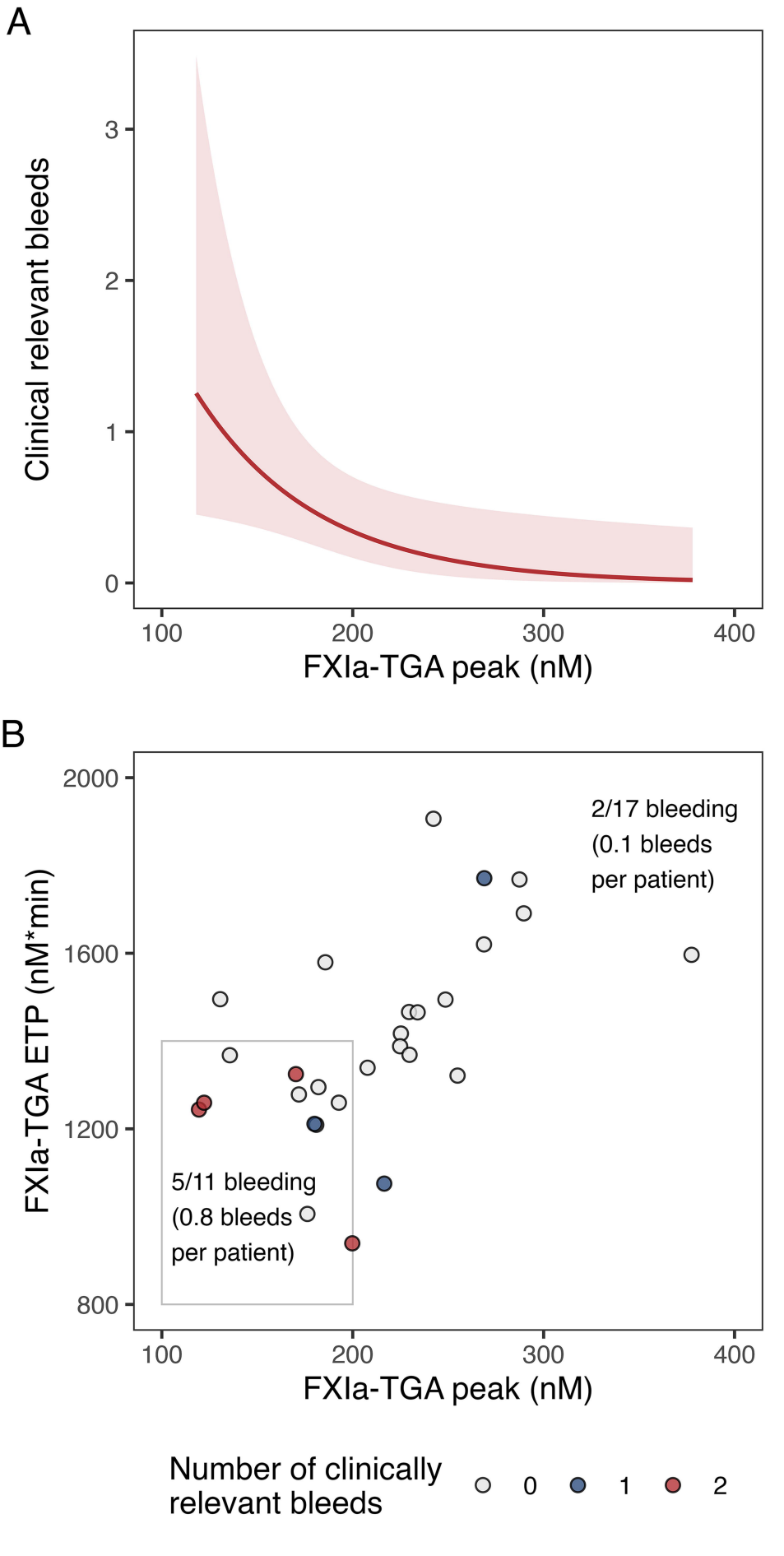

Enrollment
GTH-AHA-EMI
N = 47
End of week 1
Emicizumab loading completed
N = 47
No sample or biobanking consent
N = 19
Biomarker study
N = 28
End of week 12
Prospective observation for CRNB
N = 28


A
Emicizumab
Emicizumab (μg/ml)
50
40
30
20
10
<LOQ
B
Factor VIII
Factor VIII activity (IU/dl)
150
100
50
0
C
Factor IX
Factor IX antigen (IU/dl)
300
200
100
0
D
Factor X
Factor X antigen (mg/l)
15
10
5
0
E
Factor XI
Factor XI antigen (mg/l)
10
5
0
F
TF-TGA peak
Peak (nM)
300
200
100
0
G
TF-TGA ETP
ETP (nM*min)
2000
1000
0
H
FXIa-TGA peak
Peak (nM)
600
400
200
0
I
FXIa-TGA ETP
ETP (nM*min)
3000
2000
1000
0

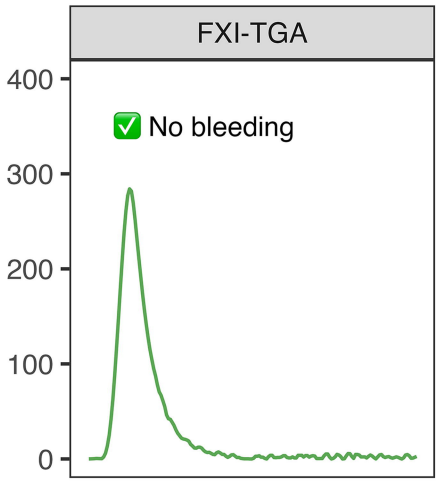

A
400
300
200
100
Peak thrombin (nM)
<LOQ
10
20
30
40
50
Emicizumab (μg/ml)

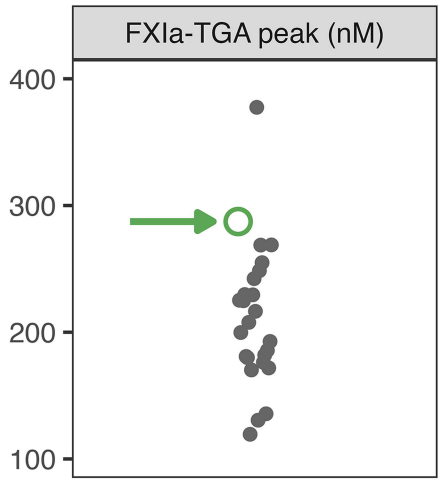

B
400
300
200
100
Peak thrombin (nM)
<LOQ
10
20
30
40
Factor VIII (IU/dl)

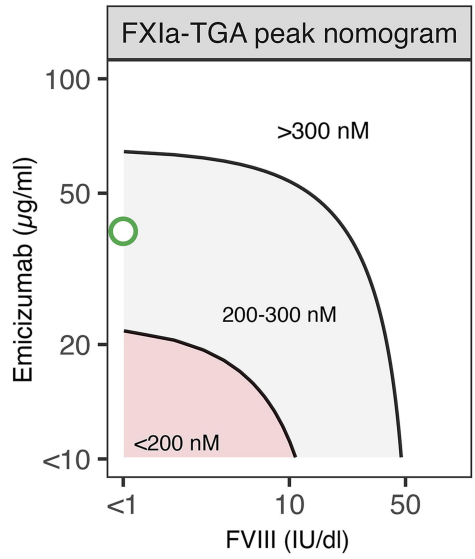

100
70
50
30
20
<10
Emicizumab (μg/ml)
>300 nM
200-300 nM
<200 nM
<1
2
5
10
20
50
Factor VIII (IU/dl)


A
Clinical relevant bleeds
0
1
2
3
100
200
300
400
FXIa-TGA peak (nM)
B
FXIa-TGA ETP (nM*min)
800
1200
1600
2000
2/17 bleeding
(0.1 bleeds
per patient)
5/11 bleeding
(0.8 bleeds
per patient)
100
200
300
400
FXIa-TGA peak (nM)
Number of clinically
relevant bleeds
0
1
2

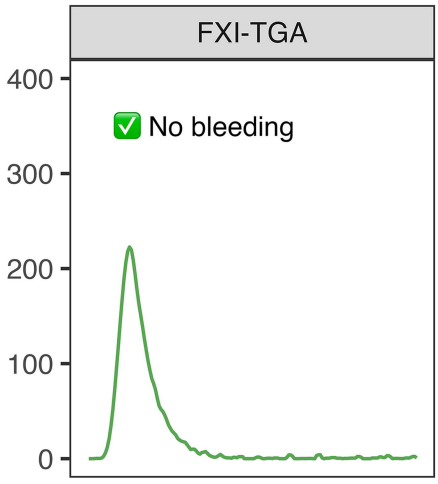
Patient 1

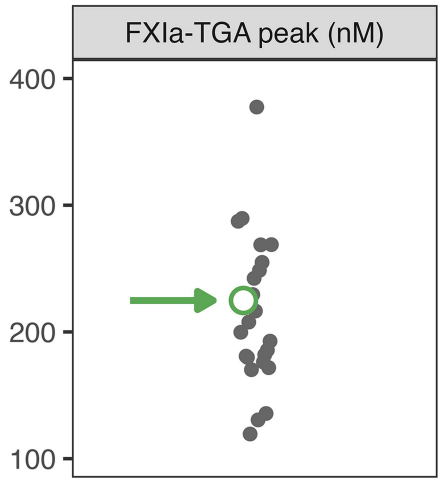


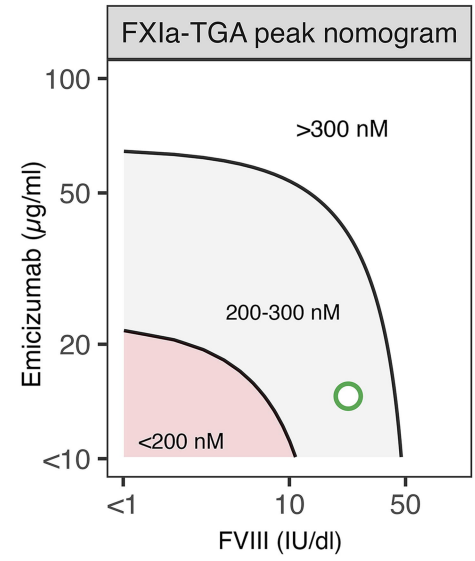




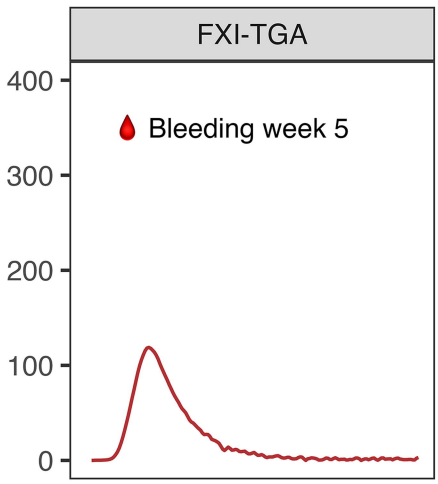
Patient 2

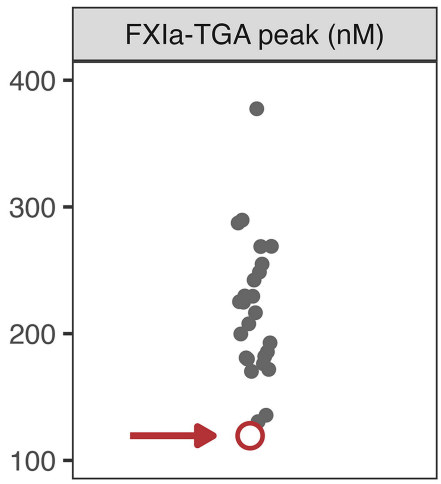


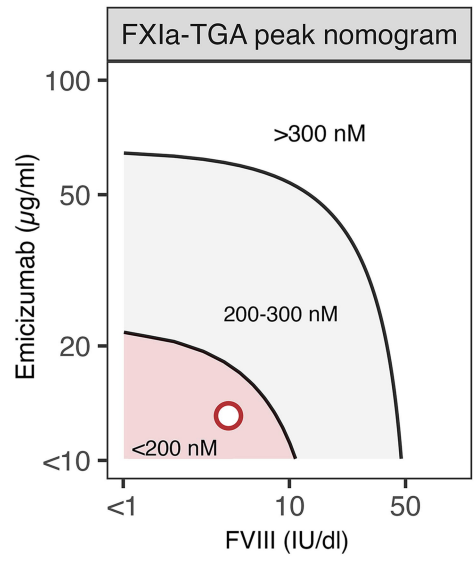




Patient 3







Supplement

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

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Patient sample collection and handling

Sampling was prospectively planned and standardized in the GTH-AHA-EMI study protocol. Venous blood was drawn using 21G needles into Sarstedt monovettes containing 1/10 (v/v) 0.109 mol/l sodium citrate. Samples were centrifuged twice at 1500 *g* for 15 min and shipped on dry ice to the central laboratory (Hannover Medical School). Storage at the central laboratory was at -80 °C until analysis. Samples were thawed at room temperature (RT) and briefly spun before analysis. Gentle thawing at RT was chosen, because thawing at 37 °C had previously been noted to result in precipitation in part of the samples.

Reference group sample collection

Plasma samples for the reference group were obtained as leftover material from routine diagnostic testing at the Central Laboratory of Hannover Medical School. Samples were age- and sex-matched to GTH-AHA-EMI patients in a 1:1 ratio. Inclusion criteria for the reference group were as follows: (1) patient presentation to the emergency department or admission unit; (2) hemoglobin concentration ≥10 g/dL on complete blood count; (3) normal prothrombin time and activated partial thromboplastin time; (4) undetectable anti-Xa activity and no reduction of other coagulation factors below the lower limit of normal, if tested; (5) residual plasma volume of at least 800 µL after completion of routine diagnostics; and (6) freezing of the residual plasma to –80 °C within 4 hours of sample collection. Samples were anonymized after collection and otherwise treated identically to patient samples. Reference ranges were calculated as mean ± 1.96 x standard deviation (SD).

Coagulation factor, emicizumab, and inhibitor assays

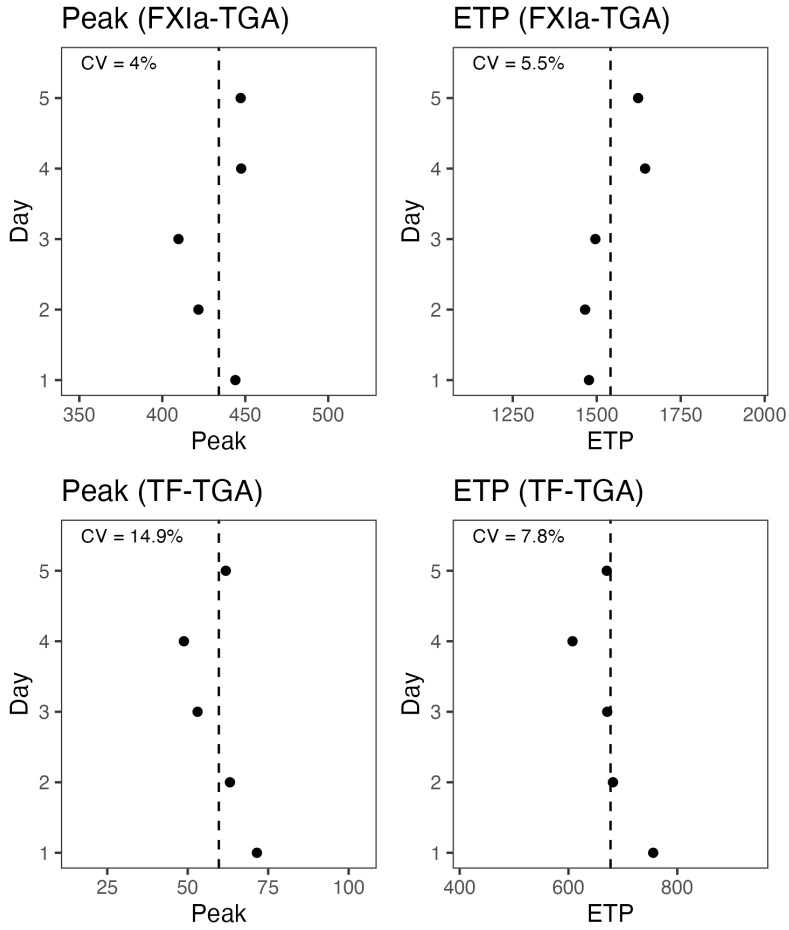
FVIII activity was determined using a chromogenic assay with bovine components (Coamatic® factor VIII, Haemochrom, Essen, Germany). Emicizumab levels were determined as previously described.[1] Factor IX, X, and XI concentrations were determined using enzyme-linked immunosorbent assays (FIX: VisuLize™ Factor IX Antigen Kit, Affinity Biologicals Inc., The Netherlands; FX: Human Factor X ELISA Kit,

Abcam BV, Amsterdam, The Netherlands; FXI: Human Factor XI ELISA Kit, Abcam). The FVIII inhibitor titer was determined in heat-inactivated plasma serially diluted in FVIII-deficient plasma and incubated with standard human plasma at 37 °C for 2 h (both obtained from Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). The residual FVIII activity was determined using a chromogenic assay with bovine components (Siemens). The inhibitor titer was calculated according to standard procedures.[2]

TGA assays

The TF-TGA and FXIa-TGA assays used in this study were performed using the Calibrated Automated Thrombogram method on a Fluoroskan Ascent instrument (CAT, Stago, Asnières-sur-Seine, France). The assay was performed according to the manufacturer's instructions by mixing 80 µl of sample with 20 µl trigger or calibrator reagent. For TF-TGA, the trigger reagent contained low amounts of TF and phospholipids (designated as *PPP low reagent* by the manufacturer). For FXIa-TGA, the trigger reagent contained FXIa (0.6 nM per reagent volume, ThermoFisher Scientific, Dreieich, Germany), diluted in phospholipids (MP reagent, Stago). After incubation at 37 °C for 10 min, the test was initiated by adding 20 µl prewarmed $CaCl_2$/substrate reagent (FluCa, Stago). The reaction was observed at 37 °C for at least 80 min. Analysis was performed using Thrombinoscope™ software (Stago).

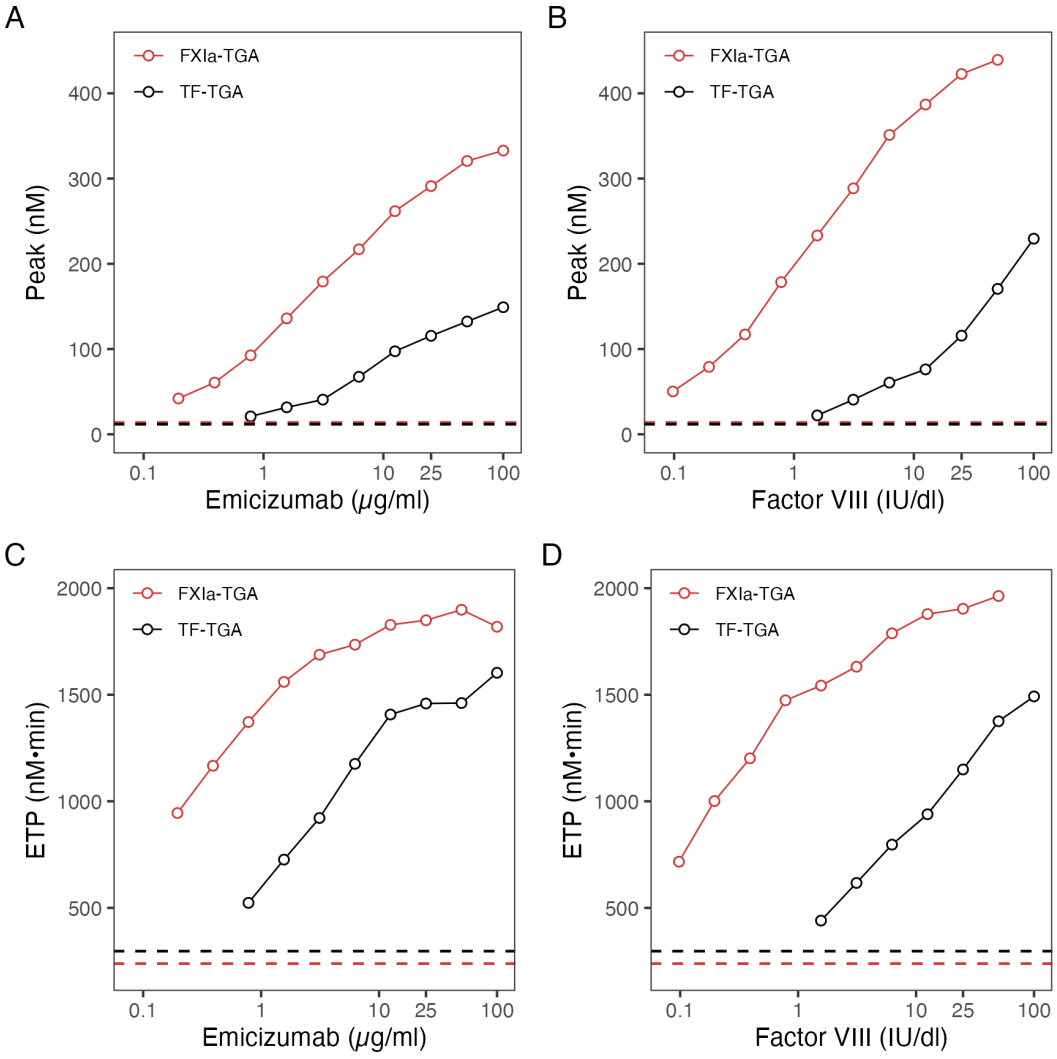
The day-to-day coefficient of variation (CV) of a control sample was <15% for peak and endogenous thrombin potential (ETP, Supplement Figure 1).



Supplement Figure 1 **Day-to-day precision of TGA measurements.** A control sample was prepared from 2 different healthy donors and stored at -80°C in aliquots of 400 µl until analysis. Measurements of FXIa-TGA and TF-TGA were conducted by two different observers across 5 working days.

The response of TF-TGA and FXIa-TGA parameters towards increasing concentrations of FVIII and emicizumab was assessed using FVIII-deficient plasma spiked with either FVIII or emicizumab (Supplement Figure 2). FXIa-triggered TGA had higher peaks compared to TF-TGA. FXIa-TGA peak was also more responsive to low concentrations of emicizumab or FVIII as compared to TF-TGA peak. There was a linear increase in TGA peaks in response to logarithmic concentrations of emicizumab (up to 100 µg/ml) or FVIII (up to 100 IU/dl). FXIa-TGA ETP was saturated already at low concentrations of

emicizumab (10 µg/ml). Therefore, peak appeared to be the more useful TGA parameter for further analysis.



Supplement Figure 2 FXIa-TGA and TF-TGA parameters according to FVIII and emicizumab standard concentrations in FVIII-deficient plasma. FXI-TGA (red) and TF-TGA (black) for standards of emicizumab and recombinant full-length FVIII spiked into FVIII-deficient PPP. (A) TGA peak according to emicizumab level. (B) TGA peak according to FVIII activity. (C) Endogenous thrombin potential (ETP) according to emicizumab level. (D) ETP according to FVIII activity. X axis (concentration) is logarithmic. Results of FVIII-deficient PPP without emicizumab or FVIII (buffer control) are indicated by dashed lines (red for FXI-TGA, black for TF-TGA). The curves represent means of two independent experiments.

TGA reference values

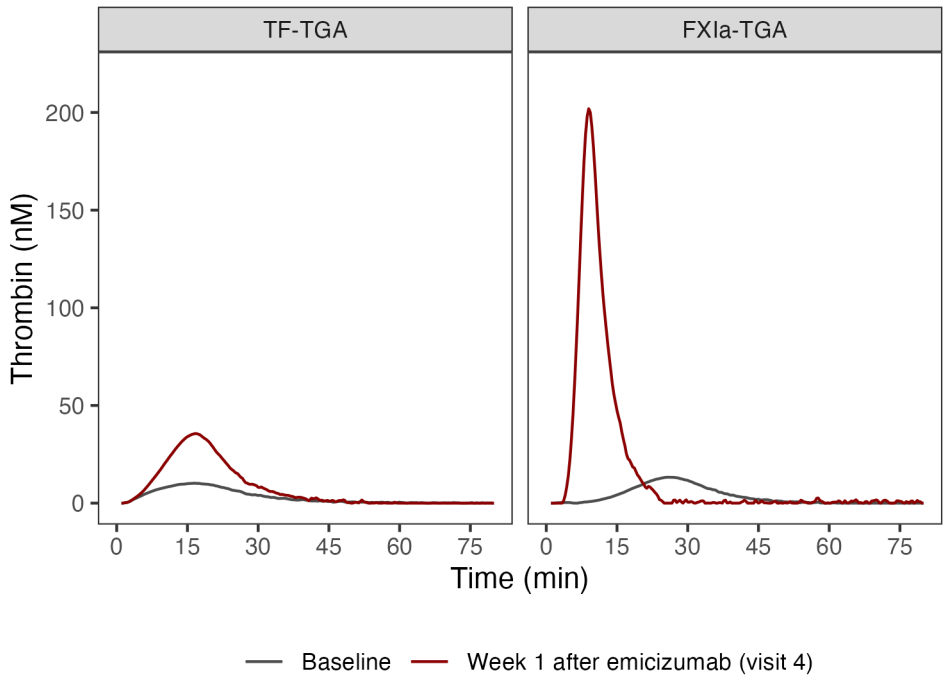
Reference values obtained for TF-TGA and FXIa-TGA parameters are shown in Supplement Table 1.

Supplement Table 1 **Reference intervals.** Data were obtained from a reference group (n=28) of patients that was matched for age and sex to GTH-AHA-EMI patients. Intervals are mean ± 1.96*SD (2.5th to 97.5th percentile).

Parameter	TF-TGA	FXIa-TGA
Peak (nM)	60.8 - 319.8	317.6 - 623.5
ETP (nM*min)	891.3 - 2269.9	1096.1 - 2719.2

TGA results before and after emicizumab loading in clinical samples

A representative patient example is shown in Supplement Figure 3.



Supplement Figure 3 **TF-TGA and FXIa-TGA in a GTH-AHA-EMI study patient before and after emicizumab loading.** Consistent with the general trend for the entire study population, emicizumab caused a higher increase in TGA peak and ETP for FXIa-TGA as compared with TF-TGA. The emicizumab level in the patient was 13.2 µg/ml in week 1 after loading (visit 4).

Descriptive laboratory data of the study population

Descriptive statistics for all laboratory parameters are shown in Supplement Table 2. A graphic representation is provided in Figure 2 of the main paper.

Supplement Table 2 Descriptive laboratory data.

Characteristic	Early after starting emicizumab N = 28[1]	Reference interval
Emicizumab activity (µg/ml)	20.9 (13.1, 31.5)	<10
	22.2 (<10-49.2)	
FVIII activity (IU/dl)	1.2 (0.9, 4.2)	50-150
	5.8 (0.5-38.5)	
Inhibitor (BU/ml)	17.5 (4.5, 109.4)	<0.6
	131.7 (0.6-1,171.9)	
FIX antigen (IU/dl)	111.4 (91.4, 153.7)	50-150
	126.3 (40.4-291.0)	
FX antigen (mg/l)	9.1 (7.5, 9.5)	7-12
	8.6 (5.1-11.4)	
FXI antigen (mg/l)	3.1 (2.6, 3.6)	2-7
	3.1 (1.2-6.2)	
TF-TGA peak (nM)	14.9 (11.0, 28.9)	61-320
	19.5 (2.5-57.7)	
TF-TGA ETP (nM*min)	292.2 (197.3, 478.9)	891-2270
	319.5 (0.0-667.7)	
FXIa-TGA peak (nM)	212.1 (178.1, 245.4)	318-624
	212.6 (119.5-377.5)	
FXIa-TGA ETP (nM*min)	1,368.2 (1259.5, 1,537.4)	1096-2719
	1,398.5 (939.2-1906.4)	

[1]Data provided as median (interquartile range) in the 1st row, and mean (range) in the 2nd row.

Effect of emicizumab and coagulation factor levels on peak thrombin generation measured by TF- and FXIa-triggered TGA assays

To analyze the association between TGA parameters, emicizumab level and coagulation factors, generalized linear modeling was applied with peak thrombin as response variable, and emicizumab and coagulation factor levels as covariates (Supplement Table 3). Effect estimates were reported with the 95% confidence intervals. Univariable models were based on z-transformed covariates to allow direct comparison of relative effect sizes; coefficients represent the change in thrombin peak (in SD units) per one SD increase in the predictor. Multivariable models were built using the original measurement scales to support interpretation in clinical units. Samples were obtained from 28 patients early after emicizumab loading.

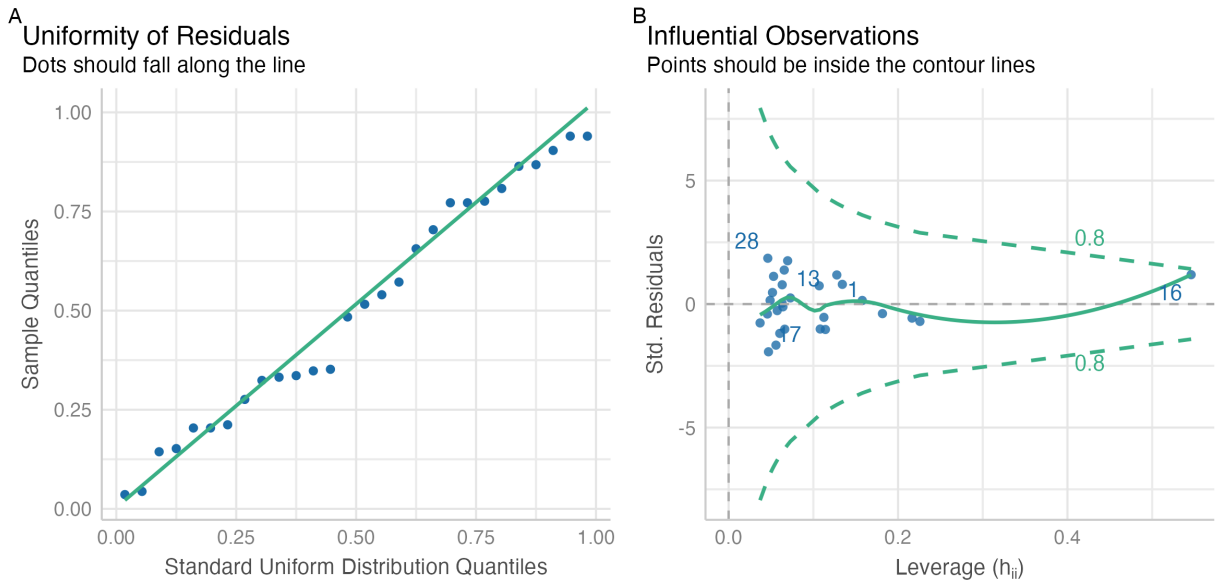
Supplement Table 3 Effect of emicizumab and coagulation factor levels on peak thrombin generation.

Effect estimates are reported with 95% confidence intervals and significance levels.

Covariate	TF-TGA	FXIa-TGA
Univariable		
Emicizumab	0.49 (0.16 - 0.82) **	0.58 (0.28 - 0.89) ***
FVIII	-0.15 (-0.52 - 0.22)	0.48 (0.15 - 0.81) **
FIX	0.01 (-0.37 - 0.38)	-0.21 (-0.58 - 0.16)
FX	-0.13 (-0.51 - 0.24)	0.24 (-0.12 - 0.61)
FXI	-0.1 (-0.47 - 0.28)	0.23 (-0.14 - 0.6)
Multivariable		
Intercept	9.07 (-0.28 - 18.42)	141.15 (108.79 - 173.51) ***
Emicizumab	0.54 (0.18 - 0.91) **	2.51 (1.26 - 3.76) ***
FVIII	-0.28 (-0.77 - 0.2)	2.71 (1.03 - 4.4) **

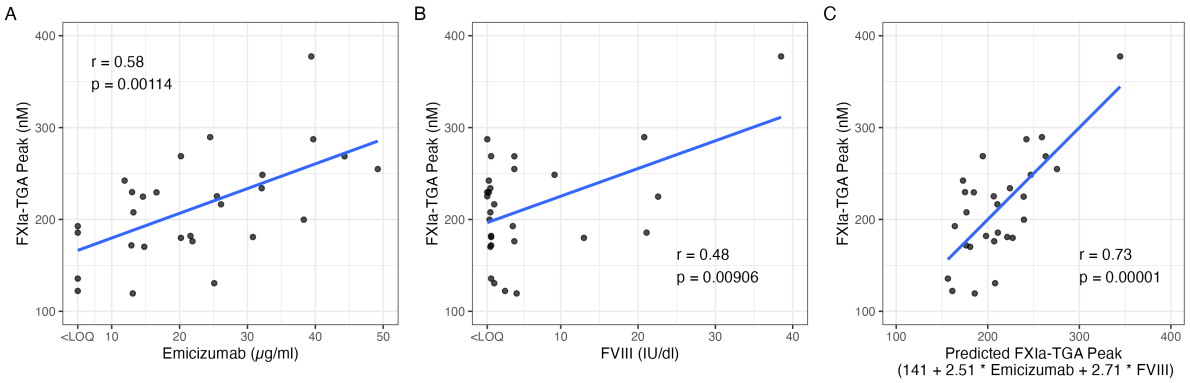
** p <0.01; *** p <0.001

Model assumptions were checked by assessing uniformity of residuals and the influence of outliers (Supplement Figure 4).



Supplement Figure 4 Diagnostic checks for the multivariable model predicting FXIa-TGA peak based on emicizumab level and FVIII activity. (A) A Q-Q plot was used to assess the distribution of residuals. (B) The influence of outliers was evaluated using a composite outlier score, as implemented in the 'check_outliers' function from the 'performance' R package.[3]

Simple correlation plots were drawn to show the relationship between TGA peak and emicizumab or FVIII level illustrating the multivariable relationship (Supplement Figure 5). Note that correlation between FXIa-TGA peak and emicizumab level or FVIII level is modest (panel A and B); however, the composite of emicizumab level and FVIII level is more closely related to FXIa-TGA peak because emicizumab and FVIII independently increase FXIa-TGA peak (panel C).



Supplement Figure 5 Correlation plots for TGA-FXIa peak versus (A) emicizumab level, (B) FVIII activity, (C) FXIa-TGA peak as predicted by the model in Supplement Table 2. LOQ denotes values below the limit of quantification.

Supplement references

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