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

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Supplement

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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

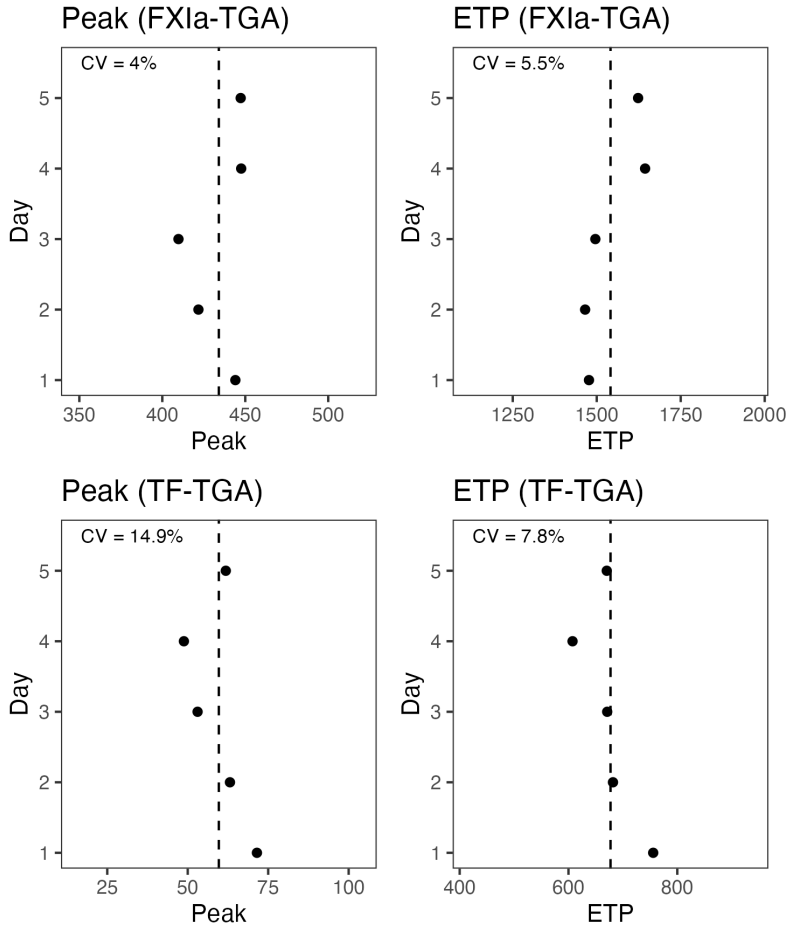
FVIII activity was determined using a chromogenic assay with bovine components (Coamatic® factor VIII, Haemochrom, Essen, Germany). Emicizumab levels were determined as previously described.[1] Factor IX, X, and XI concentrations were determined using enzyme-linked immunosorbent assays (FIX: VisuLize™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

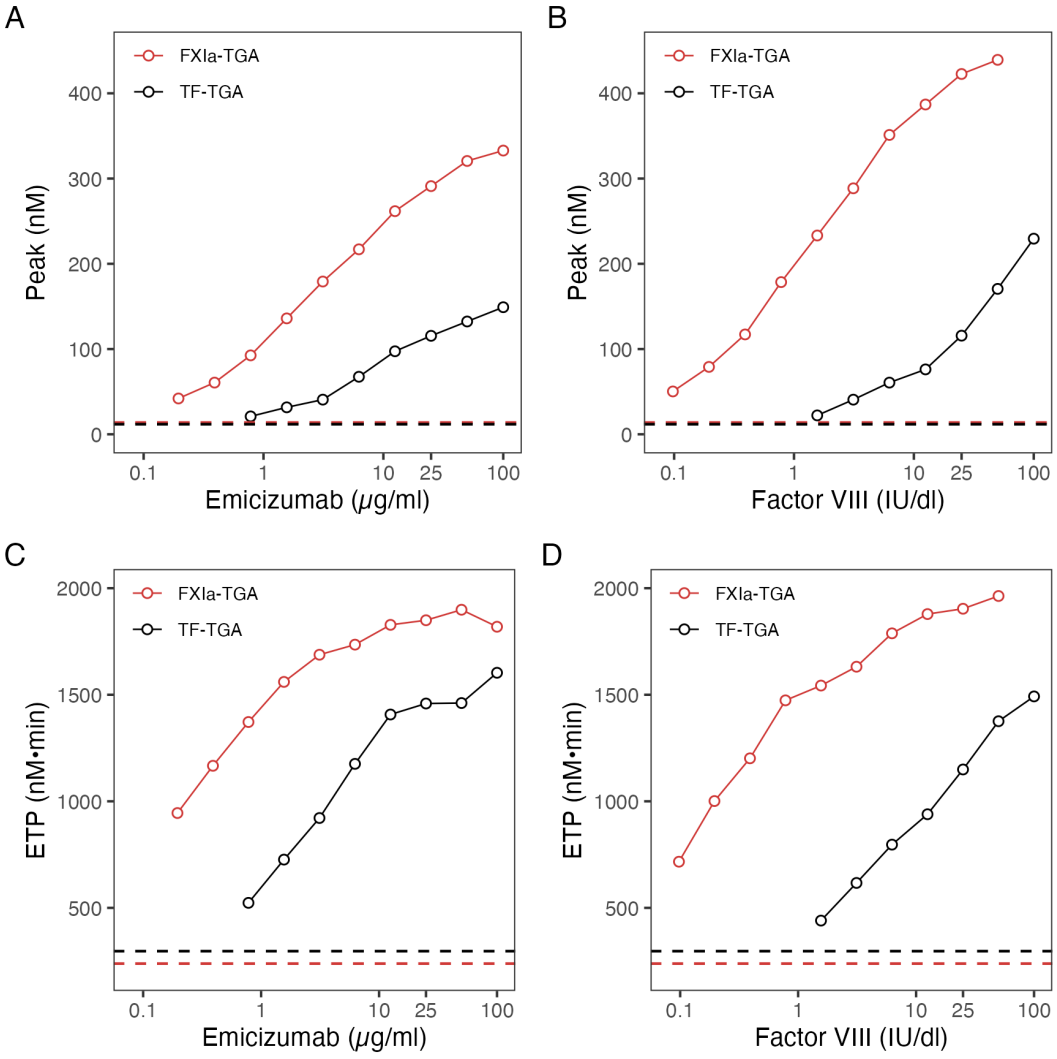
The day-to-day coefficient of variation (CV) of a control sample was <15% for peak and endogenous thrombin potential (ETP, Supplement Figure 1).



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

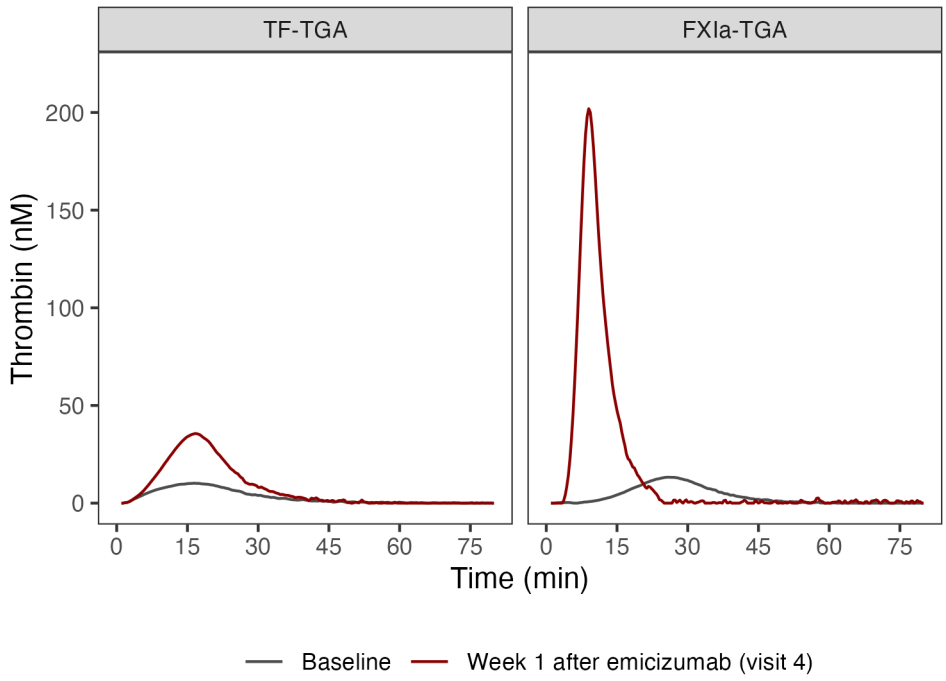
Reference values obtained for TF-TGA and FXIa-TGA parameters are shown in Supplement Table 1.

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.

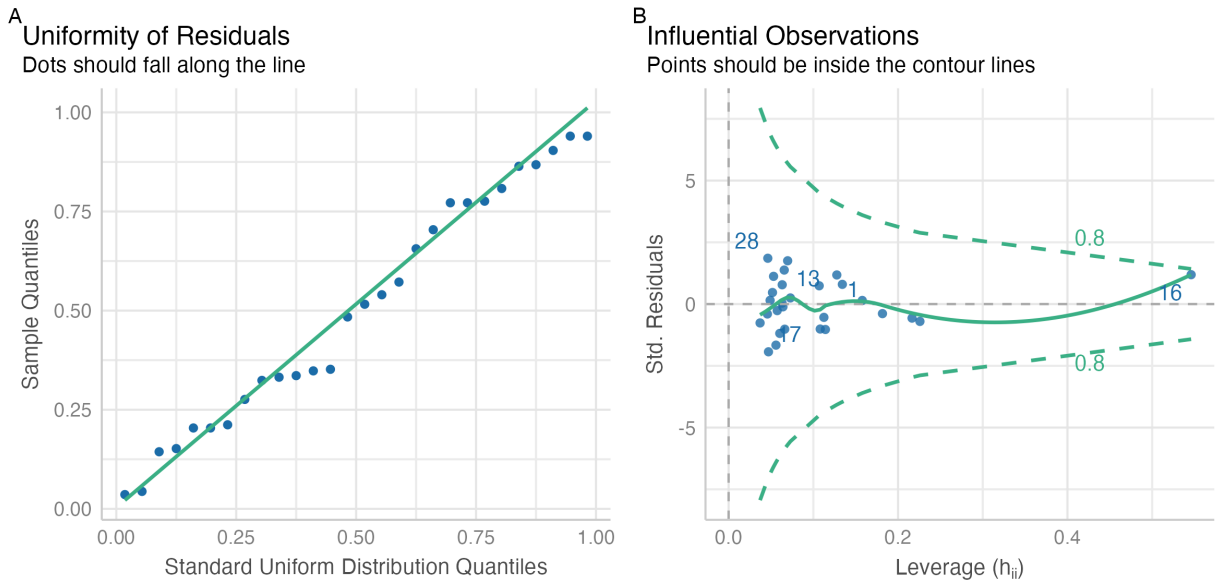
Supplement Table 3 Effect of emicizumab and coagulation factor levels on peak thrombin generation.

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) **

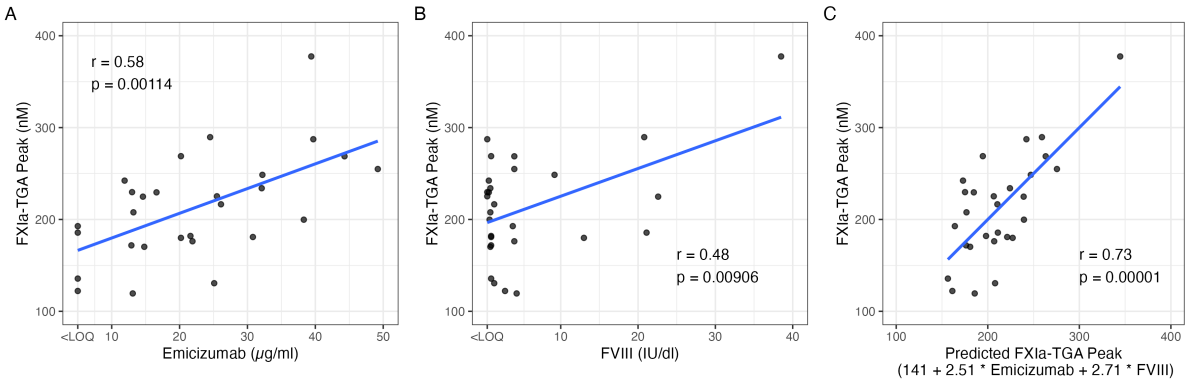
** p <0.01; *** p <0.001

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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