Supplementary data

Elevated levels of tissue factor pathway inhibitor in patients with mild to moderate bleeding tendency

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Methods

Supplementary paragraph 1 - Inclusion- and exclusion criteria

Patients aged ≥ 16 years who were referred to the hemostasis outpatient clinic of the Clinical Division of Hematology and Hemostaseology, Department of Medicine I, Medical University of Vienna, with a mild-to-moderate bleeding tendency and without a previous diagnosis of a bleeding disorder were included. Patients who had surgery or delivery within the last six weeks, bacterial infection within the last two weeks, active malignancy or pregnancy, thrombocytopenia (<100 $x10^9$/L), intake of anticoagulants/antiplatelet-/anti-inflammatory drugs (last 5-10 days) or a current acute phase reaction were not included. Also patients under continuous anticoagulant and/or antiplatelet therapy and/or with impaired liver (prothrombin time <75 % of normal due to deficiency in vitamin K-dependent clotting factors) or kidney function (GFR <60 mL/min/1.73 m^2) were not included.

Supplementary paragraph 2 - Assessment of bleeding severity

Upon study inclusion, patients and controls underwent a structured interview on the general medical and bleeding history using a standardized questionnaire. The bleeding phenotype was evaluated by two standardized bleeding assessment tools: the Vicenza bleeding score (BS) and from 2013 also the ISTH-BAT.[1,2] The interviews were performed by physicians or trained health personnel. A hemostatic laboratory assessment was performed, on the basis of which diagnoses of coagulation disorders were established or patients were categorized as patients with BUC. Within the applied standardized bleeding assessment tools, we recorded different bleeding symptoms, such as epistaxis, hematoma/easy bruising, small wound bleeding, oral mucosal and/or gingival bleeding, gastrointestinal bleeding, postpartum bleeding, muscle and/or joint bleeding, bleeding after tooth extraction, postsurgical bleeding, and menorrhagia and other bleedings in their most severe occurrence. In total, the bleeding score ranges from 0 (no symptoms) to 33 (all symptoms require medical intervention) points for the Vicenza BS [1] and 0 to 56 for the ISTH BAT,[2] respectively. Cut offs for pathological bleeding are defined as ≥3 points for males and ≥5 points for female patients for the Vicenza BS and ≥4 points for males and ≥6 points for females in the ISTH BAT. Both the Vicenza- and the ISTH BAT have a low ability to distinguish patients with an established bleeding disorder from those with BUC, as recently published.[3] We evaluated the absolute bleeding score, as well as the different bleeding manifestation sites.

Supplementary paragraph 3 - Blood sampling

Blood samples were drawn with a 21-gauge butterfly needle (Greiner Bio-One, Kremsmuenster, Austria) by antecubital venipuncture into a Vacuette tube (Greiner Bio-One, Kremsmuenster, Austria) containing trisodium citrate (nine parts of whole blood, one part of trisodium citrate 3.8%). Samples were timely processed to routine laboratory assessments and to storage at the biobank facility of the Medical University of Vienna (www.biobank.at).[4] For this project, platelet poor plasma was prepared by centrifugation at 2000g for 15 min at 15 °C (Hettich Rotanta 460 Robotic, Tuttlingen, Germany) and as a second step at 18,000g for 2 min (Eppendorf 5417R, Hamburg, Germany) and stored at <-70°C in the biobank. For DNA isolation, whole blood samples were also stored in the biobank.

Supplementary paragraph 4 - Thrombogenomics and genetic testing

Samples were processed in batches with 500ng of each sample fragmented using a Covaris E220 (Covaris Inc., Woburn, MA, USA). Samples were processed using the ROCHE KAPA HTP Library Preparation kit (Roche Diagnostics Ltd., Burgess Hill, UK).

Supplementary table 1 - Laboratory tests performed in patients and healthy controls

Screening tests and factor assays
Activated partial thromboplastin time (aPTT), seconds
Prothrombin time (PT), %
Fibrinogen-Clauss, mg/dL
Von Willebrand factor antigen (VWF:Ag), %
Von Willebrand factor ristocetin cofactor activity (VWF:RCo), %
Factor VIII activity, %
Factor IX activity, %
Factor XIII activity, %
Factor V activity in patients with PT<75% and aPTT>41 seconds
Factor VII activity in patients with PT < 75%
Factor XI activity in patients with aPTT > 41 seconds
Platelet function testing
Light transmission aggregometry (Born)
ADP (10µM), %
ADP (5µM), %
Arachidonic acid (1,6mM) , %
Collagen (10µg/ml), %
Epinephrin (5,5µM), %
Ristocetin (1,2mg/ml), %
Ristocetin (0,9mg/ml), %
Ristocetin (0,6mg/ml), %
TRAP (25µM), %
Glycoproteinexpression
CD41 (GPIIb), %
CD61 (GPIIIa), %
CD42a (GPIX), %
CD42b (GPIb-alpha), %
CD36 (GPIV, Thrombospondin), %

ADP, Adenosindiphosphat; EPI, Epinephrin; TRAP, thrombin receptor activating peptide; GPIIB, glycoprotein IIb; GPIIIa, glycoprotein IIIa; GPIX, glycoprotein IX; GPIb-alpha, glycoprotein Ib-alpha; GPIV, glycoprotein IV.

Supplementary table 2 - Patients' distribution according to diagnosis and diagnostic criteria

Diagnosis	n (%)	Criteria for diagnosis and exact number of patients
Bleeding of unknown cause (BUC)	420 (67.7%)	defined as patients with normal results in all laboratory tests on plasmatic coagulation and platelet function
VWF:Ag and/or VWF:RCo ≤50 U/mL	53 (8.6%)	defined as patients with VWF values less than or equal to 50 U/mL *18 patients (32.7%) with VWF:Ag and/or VWF:RCo <30 U/mL and 37 patients (67.3%) with VWF:Ag and/or VWF:RCo ≥30 and ≤50 U/mL*
Platelet function defect (PFD)	117 (18.9%)	Definite PFD (n=35, 29.9%), defined by abnormalities in the LTA with 2 or more agonists at least 2 different occasions Possible PFD* (n=82, 70.1%), defined by abnormal LTA aggregation curves upon stimulation with 1 or more agonists or in whom platelet function was investigated at only one occasion **includes 2 patients with additional mild factor XI deficiency (FXI ≤60 %)*
Coagulation factor deficiency (CFD)	23 (3.7%)	Factor VIII deficiency (FVIII ≤50%): n=14 (60.9%) Factor IX deficiency (FIX ≤50%): n=5 (38.5%) Factor XI deficiency (FXI ≤60%): n=3 (13.0%) Factor XIII deficiency (FX ≤10 %): n=1 (4.3%) None of our patients had FV deficiency (FV ≤60%)[5]
Hypo-/Dysfibrinogenemia	3 (0.5%)	Quantitative deficiency or qualitative defect of fibrinogen
PFD and VWF ≤50 U/mL	4 (0.6%)	Patients that had both, PFD and VWF ≤50 U/mL *For analysis all patients were allocated to both the PFD and VWF ≤50 U/mL sub-cohort*

VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor activity;

Supplementary table 3: Vicenza bleeding score of patients with high free TFPIα levels (≥95th percentile of healthy controls, ≥ 15.4 ng/mL) compared to those below this cut-off in all patients with MBDs and according to the established diagnoses

	Vicenza BS			**ISTH BAT**		
	TFPIα < 95th percentile	TFPIα ≥ 95th percentile	p	TFPIα < 95th percentile	TFPIα ≥ 95th percentile	p
	mean [±SD]	mean [±SD]		mean [±SD]	(mean [±SD])	
All patients	5.8 [±3.0]	5.8 [±3.1]	.877	6.5 [±3.5]	7.3 [±3.8]	0.204
BUC	5.7 [±2.9]	5.6 [±3.0]	.700	6.4 [±3.3]	6.9 [±3.3]	0.482
PFD	6.1 [±3.2]	5.4 [±2.7]	.458	7.0 [±4.0]	6.1 [±2.9]	0.834

TFPI, tissue factor pathway inhibitor; BS, bleeding score; BUC, bleeding of unknown cause; PFD, platelet function defect;

Supplementary table *4* Comparison of freeTFPIα levels between blood group O vs. non-O in patients and healthy controls

		Blood group O		**Blood group non-O**	**p**
	n	**median [IQR]**	**n**	**median [IQR]**	
Healthy controls	31	7.8 [3.4-11.5]	69	7.7 [4.8-10.6]	0.889
All patients*	314	8.3 [5.7-11.5]	301	8.2 [5.3-12.1]	0.858
BUC	198	8.6 [5.9-12.2]	220	8.3 [5.2-11.9]	0.521
PFD	60	9.0 [5.8-11.5]	54	8.9 [5.8-13.0]	0.538

BUC, bleeding of unknown cause; PFD, platelet function defects

* blood group data missing in 5 patients (2 in BUC subgroup, 3 in PFD subgroup)

Supplementary table 5 Correlation of free TFPIα with global coagulation tests and thrombin generation parameters in patient groups with increased TFPIα levels

	Controls	All patients	BUC	PFD
APTT, seconds	0.082 ns	-0.044 ns	-0.056 ns	0.057 ns
PT, %	0.116 ns	0.151 ***	0.177 ***	0.016 ns
TGA				
Lag time, min	0.198 ns	**0.247 *** **	**0.233 *** **	**0.350 *** **
Velocity index, nmol/L/min	-0.081 ns	-0.052 ns	-0.093 ns	-0.121 ns
Peak thrombin, nmol/L	-0.059 ns	-0.047 ns	-0.161 **	-0.147 ns
TTP, min	0.153 ns	0.144 ***	0.161 **	**0.231 ** *
AUC, nmol/L x min	0.076 ns	-0.088 *	-0.126 **	**-0.220 ** *

BUC, bleeding of unknown cause; PFD, platelet function defects; APTT, activated partial thromboplastin time ; TGA, thrombin generation assay; PT prothrombin time; TGA, thrombin generation assay; TTP, time to peak; AUC, area under the curve; Abs, maximum absorbance at plateau

Weak (r=0.2-0.4), moderate (r=0.4-0.6), strong (r=0.6-0.8) and very strong (r=0.8-1.0) correlations (Spearman)

Significance: *p<0.05; **p<0.01; ***p<0.001; ns: not significant

Supplementary table 6 Variants within the F5 gene that were found in all patients

Variant	Exon	Pathogenicity*
rs6025 cAa/cGa	10	pathogenic,benign,risk-factor,conflicting-interpretations-of-pathogenicity (FV Leiden)
rs6030 Atg/Gtg	16	uncertain significance
rs4524 aAa/aGa	13	uncertain significance
rs1046712 Ctt/Att	13	likely benign
rs13306334 Ctc/Ttc	13	likely benign
rs1800595 cAt/cGt	13	likely benign
rs4525 cAt/cGt	13	likely benign
rs6018 aAc/aCc	13	likely benign
rs6019 Gat/Cat	3	likely benign
rs6020 aGa/aAa	10	likely benign
rs6027 gAt/gGt	25	likely benign
rs6032 Aag/Gag	13	likely benign
rs6033 aTg/aCg	8	likely benign
rs9332608 Cca/Tca	13	likely benign
rs9332701 aTg/aCg	24	likely benign

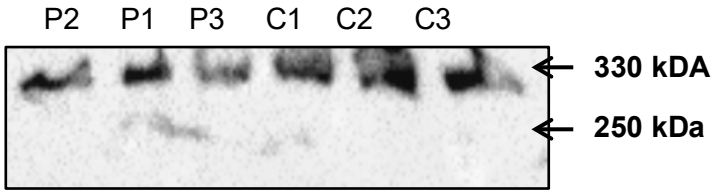
* **as stated in dbSNP**

Supplementary table *7* Clinical and laboratory characteristics of three patients with highest TFPIα values (> 40 ng/mL)

	Patient 1	**Patient 2**	**Patient 3**
Free TFPIα, ng/mL	53.7	50.0	41.5
FV short (western blot)	not detectable	not detectable	not detectable
Lag time (TGA), min	8.1	12.6	11.1
Sex	female	female	female
Age, years	62	34	59
Diagnosis	BUC	BUC	BUC
Vicenza BS	9	5	5
Number of bleeding symptoms	4	3	3
Symptoms	Small wound bleeding, bleeding after teeth extractions, post-surgical bleeding, menorrhagia	Oral mucosal bleeding, bleeding after teeth extractions, post-surgical bleeding	Easy bruising, gastro-intestinal bleeding, menorrhagia
Blood group	O	O	A
BMI, kg/m²	33.6	20.0	27.7
APTT, seconds	34.2	40.9	32.5
PT, %	94	83	110

TFPI, tissue factor pathway inhibitor; TGA, thrombin generation assay; BUC, bleeding of unknown cause; BS, bleeding score; BMI, body mass index; APTT. activated partial thromboplastin time; PT, prothrombin time

Supplementary figure 1 Immunoblot analysis of factor V in patients and healthy controls with highest free TFPIα values



Western blot analysis with an anti-factor V antibody. No variant form of factor V (Factor V short / Factor V Amsterdam, 250kDa) could be detected in the investigated patients (P1, P2, P3) and healthy controls (C1, C2, C3) with highest free TFPIα values.

References

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