Thrombin activatable fibrinolysis inhibitor pathway alterations correlate with bleeding phenotype in patients with severe hemophilia A

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Running head: TAFI pathway and bleeding in hemophilia

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Essentials

- Hemophilia A patients display varied bleeding tendency despite similar FVIII activity levels
- Factors contributing to phenotypic heterogeneity in hemophilic patients are still unknown
- We tested TAFI-related variables in hemophilia A patients with different bleeding tendency
- Impairment of TAFI activation and response to TAFIa correlated with bleeding phenotype

Abstract

Background. Patients with severe hemophilia A display varied bleeding phenotypes despite similar FVIII activity levels.

Objective. We investigated different TAFI-related variables in patients with severe hemophilia A and their possible correlation with bleeding tendency.

Patients/Methods. Sixty-one patients with severe hemophilia A (FVIII:C < 1%), treated on-demand, were included. Patients were categorized as mild, moderate and severe bleeders according to number of bleeds per year (≤2, 3-24, ≥ 25, respectively). Thirty healthy males served as controls. Fibrinolytic capacity was measured by turbidimetric assay, TAFI activation by two-stage functional assay, and response to TAFIa as the prolongation of fibrinolysis time upon addition of purified TAFIa. Circulating levels of activated TAFI (TAFIa/ai) were measured by specific ELISA.

Results. As compared to controls, haemophilic patients displayed shorter lysis time, less TAFIa generation and reduced response to TAFIa, but similar TAFIa/ai levels. Fibrinolytic capacity was similar in mild, moderate and severe bleeders, whereas TAFIa generation and response to TAFIa decreased with the increase in bleeding tendency. Unexpectedly, circulating TAFIa/ai levels were highest in severe bleeders. Patients with markedly impaired TAFIa generation or TAFIa response displayed 2-5-fold higher bleeding rate and 3-4-fold higher factor consumption than patients whose TAFI-related values approached the control ones.

Conclusion. The TAFI pathway impairment correlates with bleeding phenotype in severe hemophilia and may represent a promising tool to stratify the bleeding risk. High circulating TAFIa/ai in severe bleeders suggests that enzymes other than thrombin may induce TAFI activation in vivo.

Keywords: bleeding, CPU, hemophilia A, TAFI

Introduction

Hemophilia (A or B) is classified, based on the degree of factor deficiency, as severe (<1%), moderate (1-5%), or mild (>5-40%) (1). Approximately 40% of patients with hemophilia A have level of factor (F) VIII below 1% and are at higher risk of experiencing bleeding episodes, which often occur spontaneously or after minimal trauma (1). However, a proportion of patients with severe hemophilia, accounting for 10-15% of the total, display a mild bleeding pattern with less spontaneous bleeding and less joint damage (2, 3). The biological basis underlying these phenotypic differences remains unknown (4). Ninivaggi et al, using a very sensitive assay capable of detecting FVIII level below the usual 1% (i.e. down to 0.05%), found no correlation between the actual factor VIII level and bleeding phenotype (5). Moreover, in a 6-year follow up of patients with severe hemophilia A under prophylactic treatment, no correlation was found between trough factor VIII levels and bleeding episodes (6), suggesting that factors other than FVIII concentration contribute to the bleeding tendency. It is surmised that the type of FVIII gene (*F8*) mutation (null or non-null) or the presence of thrombophilic mutations may play a role, but definitive evidence is lacking (7). On the other hand, the use of global assays such as thrombin generation assay in plasma or thromboelastography/thromboelastometry, which gives a broader picture of the procoagulant potential, gave inconsistent results as their ability to identify hemophilic patients with different bleeding pattern (8-12).

One important consequence of impaired thrombin generation is the formation of clots that are more susceptible to fibrinolysis (13). Thrombin, indeed, enhances fibrinolytic resistance by several mechanisms, among which the activation of thrombin activatable fibrinolysis inhibitor (TAFI), a procarbixypeptidase which, once activated (TAFIa), removes the C-teminal lysines from partially degraded fibrin (13). In so doing, TAFIa reduces plasminogen binding to fibrin and hence plasminogen activation by tissue- or urokinase-type plasminogen activators (14). The impairment of TAFI activation is a major contributor to accelerated fibrinolysis in hemophilia as shown by in vitro and in vivo animal data (15-17). Moreover, Foley et al reported that the extent of TAFIa generation in blood was inversely correlated with the number of bleeds in a small group of patients (n=9) with severe hemophilia A (18), suggesting a possible clinical application of TAFI activation assay. Consistent with these findings, we found that also patients with FXI deficiency display a reduced TAFI activating capacity, which correlates with bleeding severity (19). Moreover, we observed that upon addition of purified TAFIa to the plasma of FXI-deficient patients, the inhibition of t-PA-induced clot lysis was much less pronounced as compared to normal plasma, suggesting a TAFIa resistance. The aim of the present study was to investigate the TAFI pathway in a series of severe hemophilia A patients with different bleeding phenotype. We report that the activation of TAFI and the response to TAFIa are both impaired to an extent that is significantly correlated with the bleeding severity of hemophilia A patients. Moreover, we found that the circulating levels of TAFIa and its inactive derivative (TAFIai) are increased in patient with severe bleeding tendency.

Materials and Methods

Patients

Plasma samples from 61 adult patients with severe hemophilia (FVIII:C < 1 IU dL^{-1}), enrolled in a previous study (10), were examined. Patients had no history of inhibitors and were treated exclusively on demand. Written informed consent for clinical data collection and laboratory measurements was obtained. Data on *F8* genotype, age at first bleed and first joint bleed, annual bleeding frequency and concentrate consumption were collected

from medical records and patient diaries. X-rays of elbows, knees and ankles obtained during the last 5 years prior to study entry were also evaluated by the same operator. Orthopedic examination and blood sampling were performed at enrollment. Radiologic and orthopedic joint scores were assigned according to the scoring system recommended by the Orthopaedic Advisory Committee of the World Federation of Hemophilia (20,21). *F8* defects were classified as null mutations (large deletions, inversions and nonsense mutations) or non-null mutations (small deletions/insertions, and splice site and missense mutations). Before the study was started, patients were divided into three groups based on bleeding frequency (excluding major injuries and surgical procedures) as follows: 1) mild bleeders (≤ 2 bleeds per year); 2) moderate bleeders (3-24 bleeds per year); 3) severe bleeders (≥ 25 bleeds per year). In group 2, one patient was heterozygous for FV Leiden mutation and one for G20210A prothrombin mutation. Thirty healthy age-matched controls were recruited among friends and non-consanguineous relatives who accompanied patients to visits.

Proteins and reagents

Single-chain recombinant t-PA was from Boehringer Ingelheim (Florence, Italy); human thromboplastin (Recombiplastin) was from Instrumentation Laboratory (Milan, Italy); bovine fibrinogen and potato tuber carboxypeptidase inhibitor (PTCI) were from Sigma (Milan, Italy); STA Reptilase from Stago Italia (Milan, Italy); hirudin was from Abbott GmbH (Ludwigshafen, Germany); human TAFI was from Hematologic Technologies (Essex Junction, VT, USA). Activated TAFI was prepared fresh before each experiment as reported (19).

Plasma preparation and laboratory Assays

Blood was drawn by clean venipuncture after a washout period of at least 5 days from the last concentrate infusion and collected in vacuum tubes (Becton Dickinson, Meylan, France) containing 109 mM trisodium citrate at a proportion of 9:1 (blood:anticoagulant). Blood was centrifuged within 30 min at controlled room

temperature for 20 min at 2880 · g, after which supernatant plasma was separated, divided in small aliquots, quick-frozen in liquid nitrogen and stored at -70°C until tested. At the time of blood collection, FVIII:C activity was undetectable in all but 11 samples (5 of group 2 and 6 of group 3), in which it was slightly above 1%. Because none of the investigated variables differed between patients with and without detectable FVIII:C (belonging to groups 2 and 3 only, not shown), the former were included in all analyses.

The lysis time of TF (0.5 pM, final concentration, f.c.)-induced clots challenged with t-PA (30 ng mL^{-1}, f.c.) was measured with a turbidimetric assay as previously described (22), with minor modifications (19). Fibrinolytic assay was performed both in the absence and in the presence of the specific TAFIa inhibitor PTCI (25 µg mL^{-1}, f.c.). TAFIa generation upon clotting activation with TF was evaluated by a functional, two-stage assay as reported (23). Briefly, plasma was first defibrinated by reptilase (1:50, final dilution) for 1 h at 37°C (22). Then, it was challenged with TF (0.5 pM), using mixtures identical to those used for clot lysis assay, but without t-PA, and incubated at 37°C. After 10 min, an aliquot was withdrawn, mixed with hirudin (200 U mL^{-1}, f.c.) to stop TAFI activation, and kept on melting ice until tested. TAFIa activity was evaluated as the ability to prolong the lysis time of purified fibrin clots. Thirty-five µL of sample were added to a microplate well along with 25 µL bovine fibrinogen (830 µg mL^{-1}, f.c.), 10 µL t-PA (30 ng mL^{-1}, f.c.) and 40 µL Tris-buffer, after which clot formation was induced by 10 µL reptilase (1:24, final dilution). The plate was read every minute at 405 nm at room temperature (to reduce the temperature-dependent TAFIa decay) for lysis time measurement. PTCI served as a reference for the absence of TAFI activity and results were expressed as prolongation of lysis time over the PTCI-containing sample.

The response of plasma to the antifibrinolytic activity of TAFIa (TAFIa response) was evaluated by a turbidimetric assay as reported (19). Ten µL of test plasma was added to the well of a microtiter plate along with 10 µL TAFIa (83 ng mL^{-1}, f.c.), 10 µL t-PA (30 ng mL^{-1}, f.c.), 25 µL bovine fibrinogen (830 µg mL^{-1}, f.c.), 55 µL Tris-buffer or PTCI (25 µg mL^{-1}, f.c.), and 10 µL reptilase (1:24, final dilution). The plate was incubated at room temperature, and the lysis time was calculated by the changes in optical density. The response to TAFIa was

expressed as prolongation of lysis time over the PTCI-containing sample. Control experiments revealed that no prolongation of lysis time occurred in the absence of exogenous TAFIa, ruling out the activation of endogenous TAFI.

The circulating levels of TAFIa and its inactive derivative TAFIai, collectively referred to as TAFIa/ai, were assayed by a specific ELISA (Asserachrom TAFIa/ai, Diagnostica Stago, Asnieres, France).

Due to the limited volume of plasma samples available, some assays could not be performed in all samples.

Statistical analysis

Data are reported as median and interquartile range or percent. Difference between groups was assessed by Kruskal-Wallis or Jonckheere-Terpstra trend test (continuous variables) or by Chi-squared test (categorical variables). Diagnostic performance was evaluated by Receiver Operating Characteristic (ROC) curve. To determine the difference in bleeding rate and factor consumption in patients with different degree of TAFI-related alterations, we categorized the patients into two groups using as cutoff the TAFI-related value derived from ROC curve analysis, that is the value that minimizes the number of false positives and false negatives. Statistical analyses were performed by MedCalc ®, version 17.8.6 (Mariakerke, Belgium).

Results

Main characteristics of patients

As expected, factor consumption, orthopedic joint score and Pettersson score increased significantly moving from mild to severe bleeders (table 1). Accordingly, age at first bleed and age at first joint bleed were markedly higher in patients with mild bleeding as compared to moderate and severe bleeders. The proportion of patients with *F8* null mutations was increasingly higher in patients with greater bleeding severity, even though it did not reach the statistical significance in our patient sample. The number of patients with detectable FVIII antigen did not show statistical difference among the three groups.

Table 1. Main characteristics of patients grouped according to the severity of their bleeding phenotype

	Mild (n=13)	***Moderate (n=27)***	***Severe (n=21)***	**P***
Age, years	30.1 [26-41]	39.2 [32-45]	39.2 [30-48]	0.29
Number of bleeds per year	0 [0-1.25]	10 [6-14.3]	36 [34.5-48.5]	<0.0001
Factor consumption, IU/kg/year	122 [41-158]	1108 [719-1586]	2250 [2130-3270]	<0.0001
Age at first bleed, years (13,23,17)	4 [1-6]	1 [1-2]	1[1-1]	0.0032
Age at first joint bleed, years (12,21,13)	6.5 [2.5-9]	2 [2-4]	2 [1-2]	0.0003
Orthopedic joint score (10,19,13)	2 [0-7]	11.5 [7-22]	16 [13-32]	<0.0001
Pettersson score (10,13,13)	18.5 [11-25]	28.7 [25-44]	45 [22-52]	0.002
Detectable FVIII antigen, n[%] (12,22,14)	8 [66.7]	8 [36.4]	9 [64.3]	0.13
F8 null mutation, n (%) (12,26,20)	3(25)	12 (44)	12 (60)	0.16

Data represent median [interquartile range] or percent. The number of tested patients per group is indicated in round brackets. *, for continuous variables, P was calculated by Jonckheere-Terpstra trend test.

Fibrinolysis and TAFI-related variables in patients and controls

Clots from hemophilic patients lysed much faster (shorter lysis time) as compared to control clots (table 2). When the fibrinolytic assay was performed in the presence of the TAFIa inhibitor, PTCI, the difference between

controls and patients became much smaller but remained statistically significant, suggesting that both TAFI-dependent and TAFI-independent mechanisms were behind the increased susceptibility to fibrinolysis of hemophilic clots. Accordingly, the PTCI ratio (i.e. the ratio between the lysis times in the absence and in the presence of PTCI), which is used to calculate the contribution of TAFI to fibrinolytic resistance (24), was significantly lower in hemophiliacs than in controls. Consistent with the latter finding, the amount of TAFIa activity generated 10 min after clotting activation ($TAFIa_{10min}$) was visibly lower in patients as compared to controls. Moreover, when purified TAFIa was added to plasma, under conditions that prevents the activation of endogenous TAFI, the prolongation of lysis time in hemophiliacs was less pronounced than in control plasma, suggesting a resistance to the antifibrinolytic activity of TAFIa. At variance with the in vitro findings, the circulating levels of TAFIa/ai, which reflect the extent of TAFI activation in vivo, were similar in the two groups.

Table 2. Fibrinolytic resistance and TAFI-related variables in patients with hemophilia A and controls

Assay	**Controls**	**Hemophilia A**	**P**
Lysis time, min (29, 59)	74 [64-91]	47 [38-56]	<0.0001
Lysis time + PTCI, min (29, 59)	48 [35-65]	38 [31-46]	0.020
PTCI ratio (29, 59)	1.58 [1.38-1.91]	1.15 [1.0-1.47]	<0.0001
$TAFIa_{10min}$, min (19, 40)	55 [36-85]	24.5 [13-56]	0.0046
Response to TAFIa, min (28, 58)	118 [89-166]	96.5 [56-124]	0.0054
TAFIa/ai, ng/ml (30-61)	9.6 [7.9-10.9]	8.2 [6.2-11.7]	0.13

Data represent median [IQR]. The number of tested patients per group is indicated in round brackets. P was calculated by Kruskal-Wallis test. Similar results were obtained after exclusion of patients with FVIII:C activity >1%.

Fibrinolysis and TAFI-related variables in relation to bleeding tendency

Fibrinolysis variables, i.e. clot lysis time with and without PTCI and PTCI ratio, did not differ among patients with mild, moderate or severe bleeding (table 3). On the contrary, the extent of TAFI activation and the response to exogenous TAFIa decreased with the increase of bleeding severity. Moreover, once again in contrast with the in vitro TAFIa generation data, the circulating levels of TAFIa/ai were highest in patients with severe bleeding tendency.

Table 3. Fibrinolysis and TAFI-related variables in hemophilic patients with different bleeding tendency

	Mild (n=13)	***Moderate (n=27)***	***Severe (n=21)***	**P**
Clot lysis time, min (12,27,20)	**52** [36-67]	**46** [37-52]	**49** [40-57]	0.71
Clot lysis time + PTCI, min (12,27,20)	**40** [28-56]	**40** [33-45]	**37** [30-44]	0.39
PTCI ratio (12,27,20)	**1.20** [0.99-1.54]	**1.08** [0.98-1.35]	**1.28** [1.09-1.55]	0.32
$TAFIa_{10\ min}$, min (9,17,14)	**74** [28-88]	**28** [11-60]	**16** [12-26]	0.020
Response to TAFIa, min (13,27,18)	**112** [70-131]	**100** [63-138]	**61** [48-90]	0.010
Circulating TAFIa/ai, ng/ml (13,27,21)	**7.7** [6.2-11.4]	**7.0** [5.3-9.5]	**9.6** [8.0-12.1]	0.044

Data represent median [interquartile range]. The number of tested patients per group is indicated in round brackets. See Methods for additional details. P was calculated by Jonckheere-Terpstra trend test. After exclusion of patients with FVIII:C activity above 1%, P value did not reach statistical significance for Response to TAFIa (P=0.06) and TAFIa/ai (P=0.12).

Receiver-operator characteristics (ROC) curve analysis was used to evaluate the diagnostic performance of TAFI-related variables. To that purpose, hemophilic patients were categorized as negative and positive by application of two models. Model A classified patients with mild bleeding as negative and patients of the other two groups as positive; model B classified patients with mild and moderate bleeding as negative and patients with severe bleeding as positive. Of the variables listed in table 3, only those showing statistically significant differences were examined. As shown in table 4, TAFIa generation displayed a significant discriminatory power in both models, AUC being 0.744 and 0.687 in models A and B, respectively. In contrast, response to TAFIa and plasma levels of TAFIa/ai showed a statistically significant performance only in model B (AUC of 0.730 and 0.714, respectively). *F8* null mutation approached the statistical significance only in model A.

Table 4. ROC curve analysis for variables predicting bleeding tendency in patients with severe hemophilia A

	Model A				**Model B**			
	AUC [95% CI]	*P*	Sensitivity	Specificity	AUC [95% CI]	*P*	Sensitivity	Specificity
$TAFIa_{10\ min}$ (n=40)	0.744 [0.581-0.868]	*0.005*	67.7	*77.8*	0.687 [0.521-0.824]	*0.027*	78.6	61.5
Response to TAFIa (n=58)	0.624 [0.487-0.748]	*0.16*	73.3	*61.5*	0.730 [0.597-0.838]	*0.001*	83.3	62.5
TAFIa/ai (n=61)	0.531 [0.399-0.660]	*0.73*	45.8	*69.2*	0.714 [0.584-0.822]	*0.002*	71.4	72.5
F8 Null mutation (n=58)	0.636 [0.499-0.758]	0.07	52.2	75.0	0.603 [0.466-0.729]	0.14	60.0	60.5

After exclusion of patients with FVIII:C activity above 1%, P value did not reach statistical significance for $TAFIa_{10\ min}$ in model B (P=0.17)

Table 5. Bleeds per year and factor consumption in hemophilia A groups defined by levels of biomarkers below or above the threshold derived from ROC curve analysis or by the presence of *F8* null mutation.

Variable		**Bleeds per year**		**Factor consumption**	
		n	*P*	IU*	*P*
$TAFIa_{10min}$	≤29 min (n=23)	20 [8-38]	*0.025*	2069 [938-2241]	*0.0046*
	>29 min (n=17)	10 [0.8-18]		500 [53-1206]	
Response to TAFIa	≤97 min (n=30)	26 [8-38]	*0.0006*	2094 [923-2571]	*0.0012*
	>97 min (n=28)	5.5 [1-12]		711 [159-1272]	
TAFIa/ai	≤8.9 ng/ml (n=35)	8 [2.3-18]	*0.0059*	1029 [364-2030]	*0.052*
	>8.9 ng/ml (n=26)	25 [10-36]		2109 [837-2717]	
***F8* null mutation**	No (n=31)	10 [2-28]	*0.076*	871 [221-2115]	*0.081*
	Yes (n=27)	20 [6.4-36]		1600 [1049-2234]	

Data represent median [interquartile range], *, IU/kg/year. Results were similar after exclusion of patients with FVIII:C above 1%.

Next, we evaluated the annual bleeding rate and FVIII consumption in patients grouped according to the degree of TAFI-related alterations (i.e. below or above the cutoff level derived from ROC curve analysis) (table 5). Patients with more pronounced alterations of TAFIa generation and response to TAFIa (below cutoff) had a 2- to 5-fold higher number of bleeds per year and consumed 3- to 4-fold more FVIII than patients who had less pronounced changes (above cutoff). In contrast, bleeding and factor consumption were higher in patients with

TAFIa/ai levels above the cutoff. Finally, patients with null mutations had more bleeding and consumed more FVIII than patients with non-null mutations, but the difference did not reach statistical significance.

Overall, qualitatively similar results were obtained after exclusion of patients with FVIII:C activity above 1%, in which case, because of sample size reduction, some differences did not reach statistical significance as specified in the legends to tables 2 to 4.

Discussion

Premature lysis of the haemostatic plug is considered an important mechanism contributing to bleeding in haemophilic patients (25). Broze and Higuchi were the first to show that the reduction of thrombin-mediated activation of TAFI is a major mechanism behind the accelerated fibrinolysis of clots prepared from haemophilic plasma and that improving TAFIa generation, i.e. by thrombomodulin addition, restored fibrinolytic resistance (15). Since then, the evidence on the pivotal role of TAFI in hemophiliacs has been strengthened by both in vitro and animal studies (16-19). In the present study, we investigated the TAFI pathway with three different assays: i) TAFI activation in plasma triggered with TF pM concentrations, which reflects the potential of TAFIa generation; ii) circulating levels of TAFIa/ai, which reflects in vivo TAFI activation and iii) clot lysis prolongation induced by a fixed amount of purified human TAFIa. The latter assay was developed in our laboratory to detect a possible resistance to the antifibrinolytic activity of TAFIa, which was recently observed in subjects with FXI deficiency (19). When compared to healthy controls, plasma from haemophilic patients showed lesser TAFI activation, reduced response to TAFIa and greater susceptibility to t-PA-induced lysis. More importantly, the TAFI-related variables were significantly associated with the bleeding phenotype. However, as suggested by the analyses of the ROC curves, there were notable differences among the observations stemming from the three assays. For example, TAFI activation was practically normal in mild bleeders but markedly impaired in moderate and severe bleeders, suggesting that the extent of TAFI activation in plasma might be useful to identify patients with low bleeding risk. In contrast, TAFIa response approached control values in both mild and

moderate bleeders and was clearly lower in severe bleeders, thus making its assessment a potentially useful laboratory parameter to identify patients at high bleeding risk. Similar results were obtained with circulating TAFIa/ai, whose levels were highest in severe bleeders. The possible clinical relevance of our finding is further supported by the fact that patients whose TAFI activation or TAFIa response values were below the cutoff derived from ROC curve analysis had 2-5-fold higher number of bleeds per year and 3-4-fold higher factor VIII consumption as compared to patients whose values approached the normal ones (above cutoff). Concerning TAFIa/ai levels, bleeding rate and factor consumption were found to be higher (3- and 2-fold, respectively) in patients who had values above cutoff.

Even though the clot lysis assay used in this study is sensitive to TAFI activation (24), we did not find any association between clot lysis time or PTCI ratio and bleeding phenotype. One likely explanation comes from the data of Hendriks et al (26) who showed that TAFIa inhibits fibrinolysis with a threshold mechanism, meaning that whenever the TAFIa levels are below the threshold, fibrinolysis proceeds unopposed regardless of the actual TAFIa concentration. This view is supported by the observation that the treatment with rivaroxaban or apixaban had no influence on plasma clot lysis despite that both anticoagulants significantly reduced TAFI activation (27). These in vitro findings, however, do not imply that the changes in TAFIa generation detected under our experimental conditions will have no effect on in vivo fibrinolysis, where the blood flow, the concentration of plasminogen activators and the presence of cellular factors may all influence how TAFI activation, even in the low range, will impact on fibrinolysis.

Another intriguing finding was that he circulating levels of TAFIa/ai were highest in patients with severe bleeding tendency, which is in striking contrast with the in vitro TAFI activation data. However, it should be considered that in our in vitro assay the activation of TAFI is exclusively dependent on thrombin (no t-PA was present in the system) whereas in vivo it may be driven also by other enzymes such as plasmin (28). A similar discrepancy was observed in patients under warfarin treatment who had TAFIa/ai levels higher than untreated controls despite the marked reduction of (in vitro) TAFI activation caused by the anticoagulant (29). Notably,

plasma levels of TAFIa/ai in warfarin treated-patients did correlate with circulating plasmin-antiplasmin complex (marker of plasmin formation) but not with prothrombin fragment 1+2 (marker of thrombin generation) (29), suggesting that plasmin might mediate the increased TAFIa/ai levels in subjects with defective coagulation such as warfarin-treated and haemophilic patients, at least under basal conditions in intact vessels. At the site of vascular injury, however, thrombin-induced TAFI activation is thought to play a major role in protecting the plug from premature lysis (30,31), at least in some circulatory districts. In fact, Wyseure et al (17) showed that joint bleeding in congenital haemophilic mice could be ascribed to a defective thrombin-mediated TAFI activation.

In humans, the first evidence of a correlation between TAFI activation and bleeding score was provided by Foley et al. in nine patients with severe hemophilia (16). Our study adds to these data in several aspects: i) we investigated a larger group of patients who were well characterized from both clinical and laboratory standpoints; ii) we assessed different TAFI-related variables; and iii) our experiments were performed in plasma, not in blood as in the Foley's study. The latter aspect makes our assays more suitable for clinical laboratory use.

The limited volume of plasma samples available did not allow us to collect results for all the patients included in the study. The extent of TAFI activation, in particular, could only be assessed for 40 out of the 61 patients, thereby reducing the strength of our observation. Moreover, we were unable to evaluate the amount of TAFIa generated over time. It is conceivable that a time-course of TAFI activation, which provides a better picture of TAFI pathway impairment, might display a stronger association with bleeding. In this regard, it is of interest the report of Marar et al. (32) who described a single-stage fluorescent assay which, similar to the thrombin generation assay, allows the monitoring of TAFI activation in function of time. Another issue to be considered is that the mechanism behind the resistance to the antifibrinolytic activity of TAFIa, seen in our patients, remains largely unclear.

In conclusion, our data show that the degree of TAFI pathway impairment correlates with bleeding tendency in patients with severe hemophilia A, suggesting a possible clinical application of the TAFI-related assessments made in this study. These results, however, must be taken with caution because our study was not appropriately designed to assess the discriminatory capacity of the investigated assays. Nevertheless, our findings offer new avenues for exploring alternative approaches to stratify the bleeding risk in haemophilic patients. This is particularly important if one considers that *F8* mutation type, which is usually deemed as one of the most reliable indicator of the bleeding risk, was reported to account only for a small component of phenotypic variability in a large study with more than 600 patients with hemophilia A (33).

Addendum

Mario Colucci designed and supervised the study and wrote the manuscript; Fabrizio Semeraro, Concetta T. Ammollo, Lavinia Dirienzo and Antonia Vitulli performed the research; Armando Tripodi designed the study and critically revised the manuscript; Maria E. Mancuso and Elena Santagostino supervised the clinical study management and critically revised the manuscript. All authors read the final version of the manuscript and approved it prior to submission.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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