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PLASMA FIBRIN CLOTS OF PULMONARY EMBOLISM PATIENTS PRESENT INCREASED AMOUNTS OF FACTOR XIII AND ALPHA2-ANTIPLASMIN AT 3 MONTHS' ANTICOAGULATION SINCE THE ACUTE PHASE

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Fibrin cross-linking by coagulation factor (F)XIII leads to clot stabilization. Reduced plasma FXIII levels have been reported in acute pulmonary embolism (PE) patients. We investigated the impact of anticoagulant therapy on clot-bound amounts of FXIII and α2-antiplasmin and their associations with fibrin clot properties in patients with PE. Clots generated from plasma of 18 acute symptomatic patients on admission and after a 3-month treatment with rivaroxaban were assessed off anticoagulation using mass spectrometry. Plasma FXIII and α2-antiplasmin activity were determined at the 2 time points along with thrombin generation markers, plasma fibrin clot permeability (K_s), and clot lysis time (CLT). Following anticoagulant therapy, clot-bound FXIII increased from 2.97 (interquartile range, 1.98 – 4.08) to 4.66 (3.5 – 6.9) mg/g protein and α2-antiplasmin from 9.4 (7.2 – 10.6) to 11 (9.5 – 14) mg/g protein (both $p < 0.0001$). The two parameters showed positive correlation at baseline only ($r = 0.63$, $p = 0.0056$). Similarly to clot-bound amounts, plasma FXIII (+25.8%) and α2-antiplasmin activity (+12%) increased at 3 months. Plasma FXIII activity on admission, but not after 3 months since the index PE, was associated with amounts of clot-bound FXIII ($r = 0.35$, $p = 0.043$) and α2-antiplasmin ($r = 0.47$, $p = 0.048$). At baseline, clot-bound FXIII correlated with plasma F1+2 prothrombin fragments levels ($r = 0.51$, $p = 0.03$), while clot-bound α2-antiplasmin correlated with CLT ($r = 0.43$, $p = 0.036$). At 3 months associations of clot-bound FXIII and α2-antiplasmin were abolished. This study assessed for the first time changes in the fibrin clot composition following acute PE, suggesting an increase of clot-bound and plasma FXIII and α2-antiplasmin levels after 3 months.

Key words: *factor XIII, α2-antiplasmin, fibrin clot, proteomics, thrombosis, pulmonary embolism, anticoagulant therapy, blood clotting*

INTRODUCTION

A conversion of plasma fibrinogen into fibrin catalyzed by thrombin is the final step of blood coagulation. Thrombin activates factor (F)XIII, which stabilizes the fibrin clot by cross-linking and incorporates up to 40% of the plasma α2-antiplasmin, an inhibitor of fibrinolysis, into the fibrin chains making the clot more resistant to enzymatic degradation (1). Activated FXIII (FXIIIa) cross-links also thrombin-activatable fibrinolysis inhibitor (TAFI) to fibrin (1). α2-antiplasmin is a key serine protease inhibitor of the fibrinolytic system. A lack of α2-antiplasmin activity in knockout mice was shown to prevent thrombus formation, suggesting an essential role of α2-antiplasmin in the pathophysiology of venous thromboembolism (2).

Clots formed at high thrombin or FXIII levels are tightly packed, composed of thin fibrin fibers, and relatively resistant to lysis (1, 3). Decreased amounts of FXIIIa lead to reduced clot firmness and increased bleeding in patients undergoing cardiopulmonary bypass (4). Clot firmness was restored if patients received recombinant FXIII during surgery (5). Kucher *et al.* have shown that in 71 acute pulmonary embolism (PE) patients compared to 49 patients without PE circulating FXIII subunit A but not subunit B is decreased by 13.9% (6). Moreover, FXIII antigen level was inversely associated with rates of pulmonary artery occlusion suggesting consumption of blood coagulation factors during a massive clot burden (6). Combined D-dimer and FXIII measurements provided a higher positive predictive value for PE than single tests (7).

It is known that thrombus stability determines its susceptibility to embolization (8). Mice with provoked deep vein thrombosis (DVT) supplemented with FXIII formed more stable thrombi and fibrin clots resistant to lysis resulting in reduced PE risk (9).

Previously, we have shown that a multiple enzyme digestion filter aided sample preparation (MED FASP) method combined with a Total Protein Approach allows for quantitative analysis of thousands of proteins, providing their content and concentrations

within fibrin clots (10). Our recent study has shown that clots formed from plasma of acute PE patients differed from clots of control subjects in regard to about 200 proteins, including reduced amounts of clot-bound α2-antiplasmin, α2-macroglobulin, FXIII, plasminogen, and prothrombin (11). Moreover, a specific protein composition in plasma fibrin clots of acute PE patients correlated with formation of denser clots relatively resistant to lysis (11). Interestingly, we found that several proteins unrelated to the coagulation system such as C-reactive protein (CRP) modulate the fibrin clot phenotype in acute PE patients (11).

Since reduced plasma FXIII activity is associated with acute PE (6) and it may be a potential therapeutic target, we investigated whether incorporation of FXIII and α2-antiplasmin into fibrin clots changes in patients with acute PE following anticoagulant treatment and if the extent of this reaction shows association with plasma fibrin clot properties.

MATERIALS AND METHODS

Patients

A total of 20 white patients with acute symptomatic PE were recruited from December 2016 to March 2019, all emergency admissions and reevaluated after 3-month anticoagulant treatment with rivaroxaban. Healthy age- and sex-matched individuals (n = 18) served as control. The diagnosis of PE was based on the presence of typical symptoms and positive results of high resolution spiral computed tomography. The exclusion criteria were: known cancer, pregnancy, postpartum period, high-risk PE with shock or hypotension, ischemic stroke in the past 3 months, myocardial infarction in the past 3 months, end-stage kidney disease, vitamin K antagonist use, and anti-Xa activity ≥ 0.2 IU/mL. Twenty patients, treated initially with low molecular weight heparin according to the current guidelines (12) were discharged on rivaroxaban and reevaluated after 3 months of treatment, while off anticoagulant therapy. Patients with plasma concentrations of rivaroxaban > 30 ng/ml were excluded from the final analysis (13). A PE severity index (PESI) was assessed (12). The diagnosis of DVT was established by a positive finding of colour duplex sonography (visualization of an intraluminal thrombus in calf, popliteal, femoral or iliac veins) performed within the first 48 hours since enrolment. In this study unprovoked venous thromboembolism (VTE) episode was defined as having no surgery requiring general anesthesia, major trauma, plaster cast or hospitalization in the past month.

The Jagiellonian University Ethical Committee approved the study, and participants provided informed written consent.

Laboratory investigations

Blood samples were drawn from an antecubital vein with minimal stasis before initiation of anticoagulant therapy on admission and after 3 months of anticoagulant therapy with rivaroxaban 24 – 28 hours since the last dose. Blood cell count, glucose, creatinine, lipid profile, and D-dimer levels were assayed by routine laboratory techniques in the hospital laboratory. Fibrinogen was determined using the Clauss method. CRP was measured using immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Plasma α2-antiplasmin activity was evaluated by a chromogenic assay (Berichrom, Siemens Healthcare Diagnostics, Marburg, Germany), while α2-antiplasmin antigen levels were measured using an in-house ELISA that detects all forms of α2-antiplasmin and is not influenced by the presence of plasmin-antiplasmin complexes (reference range: 48 – 85 mg/l) (14). Factor XIII activity (FXIII) was measured by ammonia release assay (15), using a commercially available reagent kit (REA-chrom FXIII kit, Reanal-ker, Budapest, Hungary; reference range: 69 – 143%). Immunoenzymatic assays were used to determine F1+2 prothrombin fragments (Siemens, Marburg, Germany), thrombin-antithrombin complex (TAT, Siemens) levels, and TAFI activity (Hyphen-Biomed, Neuville-Sur-Oise, France). Anti-Xa activity was measured at baseline and at 3 months to confirm the absence of anticoagulant effects of heparin and rivaroxaban by the anti-factor Xa chromogenic assay (Biophen DiXaI, Hyphen Biomed, Neuilly-sur-Oise, France).

Plasma clot mass spectrometry

Plasma clot proteomics was performed as previously described (10). Briefly, to 100 µL of citrated plasma, 20 mmol/L calcium chloride and 1 U/mL human thrombin (Merck, Kenilworth, NJ, USA) was added. This mixture was placed into tubes which after 120 minutes of incubation were connected to a reservoir of a buffer (0.05 mol/L Tris HCl, 0.1 mol/L NaCl, pH 7.5) to rinse a fibrin gel for one hour to remove plasma proteins and heme catabolism products that had not been incorporated into the formed clots (10). The clots were lysed in a buffer consisting of 0.1 M Tris-HCl, pH 8.0, 1% sodium dodecyl sulfate and 50 mM dithiothreitol at 96ºC for 10 min. Quantitative proteomics of fibrin clots prepared *ex vivo* from citrated plasma was performed using two enzymes endoproteinase LysC and trypsin for protein digestion in Multienzyme Digestion-Filter Aided Sample Preparation (MED FASP) (16) with disulfide bridges reduction and maintaining cysteine reduced during the whole sample preparation procedure (17). Aliquots containing 1 µg peptide mixture were analyzed using a QExactive HF mass spectrometer (Thermo-Fisher Scientific, USA). MS data were searched using MaxQuant (18).

Fibrin permeation analysis

Fibrin clot permeation was determined using a pressure-driven system (10). Briefly, 20 mM calcium chloride and 1 U/mL human thrombin (Merck KGaA, Darmstadt, Germany) were added to 120 µl citrated plasma. After 2 hours of incubation in a wet chamber, tubes containing the clots were connected *via* plastic tubing to a reservoir of a buffer (0.01 M Tris, 0.1 M NaCl, pH 7.5) and its volume flowing through the gels was measured within 60 minutes. A permeation coefficient (K_s) was calculated from the equation: $K_s = QxLx\eta/txAx\Delta p$, where Q is the flow rate; L, length of the fibrin gel; η, viscosity of the liquid (in poise); A, the cross-sectional area (in cm^2) and Δp, a differential pressure (in $dyne/cm^2$).

Plasma clot lysis assay

To assess efficiency of clot lysis, clot lysis time (CLT) was used. Briefly, citrated plasma was mixed with 15 mM calcium chloride, 0.5 U/mL human thrombin (Merck), 15 µM phospholipid vesicles (Rossix, Molndal, Sweden) and 20 ng/mL recombinant tPA (rtPA, Boehringer Ingelheim, Germany) (19). The mixture was transferred to a microtiter plate and its turbidity was measured at 405 nm at 37ºC. CLT was defined as the time from the midpoint of the clear-to-maximum-turbid transition, which represents clot formation, to the midpoint of the maximum-turbid-to-clear transition (representing the lysis of the clot).

Statistical analysis

Variables are presented as numbers (percentages), mean ± standard deviation (SD) or median and interquartile range (IQR)

as appropriate. Categorical variables were compared by the Pearson's chi-squared test or Fisher's exact test. Normal distribution was assessed by Shapiro-Wilk test. The determination coefficients (r^2) were calculated to test the association between 2 variables. Differences between 2 groups were compared using the Student's test for normally distributed continuous variables and for non-normally distributed continuous variables the Mann-Whitney U test was used. For paired data the Student's t test or the Wilcoxon signed-rank tests were used as appropriate. A two-sided p-value < 0.05 was considered statistically significant. All statistical analysis were performed using STATISTICA software Version 12.5 (StatSoft STATISTICA™, Poland).

RESULTS

We evaluated 18 patients aged 64 (55 – 71) years with acute PE on admission and after 3 months treatment with rivaroxaban (15 mg/day in 2 subjects and 20 mg/day in the remainder). Two patients were excluded from the final analysis due to plasma rivaroxaban concentrations > 30 ng/ml at 3 months control visit to avoid an influence of this drug on fibrin clot phenotype (20). Patients did not differ from controls in terms of demographic variables except for 29% higher body-mass index in the former group (*Table 1*).

Pulmonary embolism patients on admission

At baseline, PE patients were characterized by 20.1% lower plasma FXIII activity, 9.1% lower plasma α2-antiplasmin activity, 14.8% higher fibrinogen and increased levels of thrombin generation markers, such as F1+2 (+172.3%) and TAT (+276.9%) as compared to healthy controls. We observed K_s reduced by 39% and CLT prolonged by 38.2% in PE patients compared to healthy controls, while plasma α2-antiplasmin antigen and TAFI activity remained unchanged (*Table 1*).

The amount of clot-bound FXIII in PE patients was 2.97 (IQR 1.98 – 4.10) mg/g protein (0.31% of the total clot mass), while of the α2-antiplasmin 9.4 (7.2 – 10.6) mg/g protein (0.99% of the total clot mass). There were no differences in clot proteomics related to PE severity, provoked versus unprovoked VTE or isolated versus PE combined with DVT (all p > 0.05). Clot-bound α2-antiplasmin was associated with clot-bound FXIII amounts ($r^2 = 0.39$, p = 0.0056).

Clot-bound FXIII in PE patients correlated positively with plasma F1+2 levels ($r^2 = 0.26$, p = 0.03), plasma FXIII ($r^2 = 0.15$, p = 0.043), and inversely with TAFI activity ($r^2 = 0.27$, p = 0.03). Clot-bound α2-antiplasmin was positively associated with plasma FXIII levels ($r^2 = 0.22$, p = 0.048), CLT ($r^2 = 0.18$, p = 0.036), and negatively with plasma TAFI activity ($r^2 = 0.26$, p = 0.03), but not with K_s.

Follow-up

The proteomic analysis showed that after 3-month anticoagulation with rivaroxaban (median plasma concentration, 13 (9-24) ng/ml) in PE patients median amount of clot-bound FXIII was increased by 57.1% (*Fig. 1A*), accompanied by 16.8% increased clot-bound α2-antiplasmin (*Fig. 1B*) compared to the baseline values. However, the clot-bound amounts of FXIII and α2-antiplasmin did not reach concentrations of both proteins observed in control clots (*Fig. 1A* and *1B*). Plasma levels of FXIII (+25.8%) and α2-antiplasmin activity (+12%) increased in PE patients after 3 months of anticoagulant therapy to the levels observed in healthy controls (*Table 1*). In PE patients we found

Table 1. Baseline characteristics of patients with pulmonary embolism (PE) at baseline and after 3-month follow-up compared to healthy controls.

	Healthy controls (n = 18)	**Acute PE (n = 18)**	**3-month follow-up (n = 18)**
Age, years	63 (48–69)	64 (55–71)	
Body-mass index, kg/m^2	24.8 (21.1–25.7)	32 (27.7–34.1)*	
Male, n (%)	8 (44.4)	9 (50)	
Comorbidities and medications			
Time from PE symptom onset, days	–	5 (3–6)	
First ever PE, n (%)	–	11 (61.1)	
Concomitant DVT, n (%)	–	6 (33.3)	
Coronary heart disease, n (%)	–	5 (27.8)	
Hypertension, n (%)	–	15 (83.3)	
Heart failure, n (%)	–	1 (5.6)	
Aspirin use, n (%)	–	6 (33.3)	4 (22.2)
Laboratory investigations			
Factor XIII activity, %	130 (118.3–139.3)	103.9 (81.9–118.3)*	130.7 (116.5–146.1)#
α2-antiplasmin activity, %	110 (103–114)	100 (91–107)*	112 (106–117)#
α2-antiplasmin, mg/L	61.4 (57.2–70.3)	62.5 (51.9–70.5)	66.4 (60.9–70.9)
Fibrinogen, g/L	3.25 (2.91–3.55)	3.73 (3.21–4.87)*	3.29 (2.96–4.33)
High-sensitivity C-reactive protein, mg/L	1.4 (0.7–1.6)	19.6 (12.4–44.8)*	2.0 (1.3–3.5)#
D-dimer, ng/mL	317 (270–457)	4011 (2406–6486)*	391 (248–547)#
Thrombin activatable fibrinolysis inhibitor activity, %	88.7 (81.2–90.8)	93.2 (87.7–99.9)	92.1 (89.3–100.2)
Prothrombin fragments F1+2, pM	119 (86–147)	324 (211–460)*	138 (102–176)#
Thrombin-antithrombin complex, µg/L	3.07 (2.43–4.12)	11.57 (4.97–19.15)*	<2.0 (< 2.0–2.58)#
Fibrin clot permeability, $\times 10^{-9}$cm^2	7.76 (7.34–8.26)	4.73 (3.61–6.75)*	6.55 (5.93–7.14)#
Clot lysis time, min	89 (76–101)	123 (108–132)*	102 (83–111)#

*p < 0.05 for PE patients on admission compared to healthy controls; #p < 0.05 for PE patients on admission compared to 3-month follow-up.

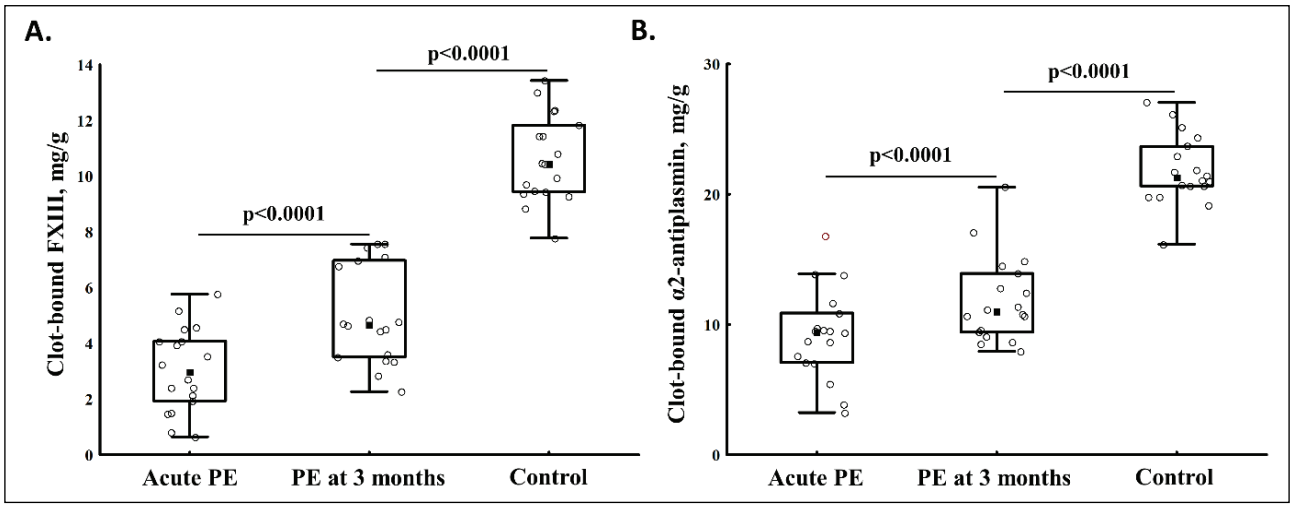

Fig. 1. Clot-bound FXIII and α2-antiplasmin amounts measured by proteomics in PE patients at baseline and after 3-month follow-up, compared to age- and sex-matched healthy controls.

no difference in fibrinogen levels or TAFI activity after 3-month rivaroxaban treatment, while CRP, D-dimer, F1+2 prothrombin fragments, and TAT levels decreased when compared to the baseline values (*Table 1*). After 3 months of treatment with rivaroxaban we observed K_s improved by 38.5% and 17.1% shortened CLT compared to baseline (*Table 1*). Associations of clot-bound FXIII or clot bound α2-antiplasmin amounts were abolished after 3-month anticoagulation.

DISCUSSION

This study shows for the first time that clot-bound amounts of both FXIII and α2-antiplasmin increased along with plasma activity of these proteins after anticoagulant treatment following acute PE episode. Since α2-antiplasmin is incorporated to fibrin by FXIIIa (1), we demonstrated using proteomic analysis, that in acute thrombosis the amounts of the two proteins in plasma fibrin clots are positively correlated. However, after 3-month follow-up, when activation of blood coagulation returns to normal, such association cannot be found using this refined methodology. Looking for plasma-derived factors affecting the incorporation of the two proteins into fibrin, we found associations of clot-bound FXIII and α2-antiplasmin with plasma FXIII and TAFI activity. This study provides new insights into the modulating role of clot-bound FXIII and α2-antiplasmin during acute thrombosis and recovery in patients following PE.

Our observations are in line with the paper by Kucher *et al.* (6), suggesting that a drop of FXIII and α2-antiplasmin levels during acute PE is associated with increased accumulation of these proteins within thrombi occluding the pulmonary arteries. However, we observed in acute PE patients that reduced clot-bound amounts of FXIII and α2-antiplasmin are associated with lower plasma activity of these proteins. It might be speculated that despite lower clot-bound amounts of cross-linking FXIII and antiplasmin, fibrin clot phenotype of acute PE patients is more prothrombotic compared to that determined at 3 months' follow-up due to increased thrombin formation resulting in denser clots, enhanced inflammatory state and potentially oxidative modifications of coagulation proteins, in particular fibrinogen (21). Moreover, our preliminary data indicate that plasma 8-isoprostane levels, as a marker of oxidative stress, are about 3.5-fold higher in acute PE patients on admission compared to 3-month follow-up (Zabczyk *et al.*, unpublished data). CRP has been shown to bind to fibrin(ogen) (22) and thus it might unfavourably influence fibrin clot properties, as demonstrated in inflammatory diseases such as chronic obstructive pulmonary disease (23), rheumatoid arthritis (24), and acute coronary syndrome (25). Recently, we have provided the first proteomic evidence that small amounts of CRP are detectable in some subjects within fibrin clots prepared from human plasma (10). Of note, in acute PE patients, it has been reported by our group that low K_s ($\leq 3.83 \times 10^{-9}cm^2$) is related to increased clot-bound CRP (11), however, the mechanism of this interaction remains to be elucidated. Taken together, we suggest that increased thrombin generation, oxidative stress, and inflammation are potent factors, strong enough to unfavorably modulate fibrin clot properties, regardless of FXIII and α2-antiplasmin consumption.

Our study is the first to show that FXIII plasma activity returned to normal after 3 months since the acute PE. Moreover, the current study showed for the first time that plasma FXIII activity correlated with clot-bound FXIII and α2-antiplasmin amounts. The α2-antiplasmin that accounts for about 90% of plasmin inhibition *in vivo* regulates fibrinolysis by forming a complex plasmin-α2-antiplasmin, inhibiting plasminogen binding to fibrin, and making fibrin more resistant to lysis *via* cross-linking with FXIIIa (26). It is not known to what extent the α2-antiplasmin incorporation into fibrin/fibrinogen differs between individual subjects and whether the degree of α2-antiplasmin incorporation modulates the fibrinolytic response *in vivo*. In our study there were no differences in fibrinogen concentrations or plasma α2-antiplasmin antigen levels between PE patients during acute phase of the disease and after 3 months, however, plasma α2-antiplasmin activity and clot-bound α2-antiplasmin amounts increased together with clot-bound FXIII levels after 3 months since the index acute PE, indicating tendency to clot stabilization with time and/or anticoagulant therapy. It has been proposed that incorporation of α2-antiplasmin into circulating fibrinogen prior to initiation of blood clotting may play an important role in down-regulating fibrinolysis (27) but we suggest that the final clot composition is regulated by other mechanisms. A role of α2-antiplasmin in VTE has been recently highlighted by our studies showing elevated α2-antiplasmin activity in association with denser fibrin clots in patients with post-discharge VTE despite thromboprophylaxis during hospital stay (28) and in patients who experienced DVT following lower limb trauma (29). It has been proven that

reduced α2-antiplasmin activity can improve thrombolysis and thrombus dissolution, being a potent target for novel thrombolytic therapies (30-33). Interestingly, the effect of α2-antiplasmin inactivation on thrombus disintegration in a mouse model was comparable to this observed after rtPA injection (31). Robinson *et al.* (34) have shown that such activity declined with thrombus aging. Therefore, it has been suggested that inhibition of FXIIIa can exert a similar effect to that of α2-antiplasmin inhibition leading to improved thrombus dissolution. However, clinical studies are highly required to corroborate such therapeutic strategy based on modulation of FXIIIa.

In our study, amounts of clot-bound FXIII correlated with plasma F1+2 levels in PE patients. This association is not surprising since thrombin activates FXIII. Our recent study showed that clots prepared from plasma of acute PE patients compared to healthy controls are characterized by 88% lower amounts of prothrombin followed by decreased amounts of antithrombin, plasminogen, FXIII or α2-antiplasmin (11). It should be highlighted that such clots are formed from plasma of acute patients in whom a consumption of blood coagulation factors occurs at the thrombus site and the plasma clot model can reflect a post-thrombotic state. We have reported that plasma clots of acute PE patients present the prothrombotic phenotype (35), which is improved at 3 months. It remains to be elucidated whether such improvement is associated with resolution of the acute phase of thrombosis or rather anticoagulant treatment used, especially that even after 3-month therapy clot protein composition still differed from healthy controls. This might indicate that patients following PE have modified fibrin clots or require longer anticoagulant treatment.

This study has some limitations. First, the sample size was limited, however, both plasma and clots prepared from plasma were obtained from a representative group of acute PE patients. Due to rivaroxaban concentrations > 30 ng/ml substantially improve fibrin clot properties, including K_s and CLT rendering them less prothrombotic (20), 2 of the 20 patients were excluded from the final analysis. Secondly, assessment of fibrin clots prepared from platelet-poor plasma eliminated the potential influence of platelets or blood cells on clot properties. Third, to remove most non-covalently bound proteins from fibrin, fibrin clots were extensively washed prior to enzymatic digestion and proteomic analysis, however, some proteins could be still bound following this procedure, which might confound the data interpretation. This study was hypothesis-generating and possible cause-effect relationships can be established on larger cohorts.

In conclusion, our study suggests that anticoagulant treatment following acute PE influences not only plasma activity of FXIII and α2-antiplasmin but also their clot-bound amounts, which might be associated with improved fibrin clot properties. Further studies are needed to clarify to what extent the change observed at 3 months is associated with anticoagulation or the natural course of the disease. Given potential new therapeutic options affecting FXIII and/or antiplasmin, the present findings appear to be relevant and deserves further research.

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