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Antithrombin Deficiency Is Associated with Prothrombotic Plasma Fibrin Clot Phenotype

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Abstract

Background Deficiency of antithrombin increases risk of venous thromboembolism. We hypothesized that antithrombin deficiency affects fibrin clot structure and function.

Methods We evaluated 148 patients (age: 38 [32–50] years; 70% women) with genetically confirmed antithrombin deficiency and 50 healthy controls. Fibrin clot permeability (K_s) and clot lysis time (CLT) along with thrombin generation capacity were assessed before and after antithrombin activity normalization in vitro.

Results Antithrombin-deficient patients had lower antithrombin activity (−39%) and antigen levels (−23%) compared with controls (both $p < 0.01$). Prothrombin fragment 1 + 2 levels were 26.5% higher in patients with antithrombin deficiency than in controls along with 94% increased endogenous thrombin potential (ETP) and 108% higher peak thrombin (all $p < 0.01$). Antithrombin deficiency was associated with 18% reduced K_s and 35% prolonged CLT (both $p < 0.001$). Patients with type I ($n = 65$; 43.9%) compared with type II antithrombin deficiency ($n = 83$; 56.1%) had 22.5% lower antithrombin activity ($p < 0.001$) and despite similar fibrinogen levels, 8.4% reduced K_s, 18% prolonged CLT, and 30% higher ETP (all $p < 0.01$). Reduced K_s was associated with lower antithrombin antigen level ($\beta = -6.1$, 95% confidence interval [CI]: −1.7 to −10.5), while prolonged CLT was associated with lower antithrombin antigen ($\beta = -69.6$, 95% CI: −9.6 to −129.7), activity ($\beta = -2.4$, 95% CI: −0.3 to −4.5), higher PAI-1 ($\beta = 12.1$, 95% CI: 7.7–16.5), and thrombin-activatable fibrinolysis

Keywords
- antithrombin deficiency
- fibrin clot
- fibrinolysis
- thrombin generation
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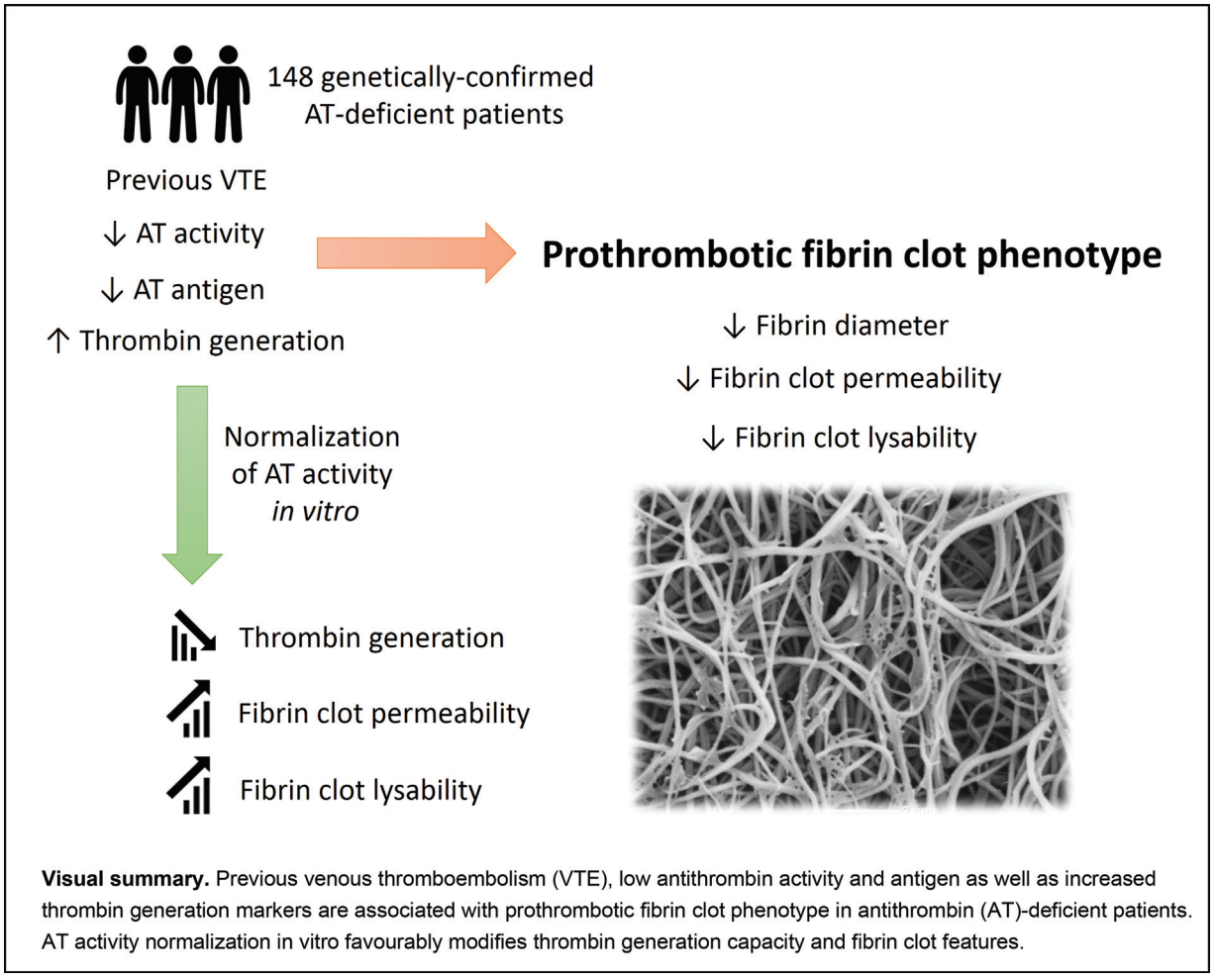

Visual summary. Previous venous thromboembolism (VTE), low antithrombin activity and antigen as well as increased thrombin generation markers are associated with prothrombotic fibrin clot phenotype in antithrombin (AT)-deficient patients. AT activity normalization in vitro favourably modifies thrombin generation capacity and fibrin clot features.

inhibitor levels ($\beta = 3.8$, 95% CI: 1.9–5.7). Addition of exogenous antithrombin reduced ETP (−42%) and peak thrombin (−21%), and improved K_s (+8%) and CLT (−12%; all $p < 0.01$).

Conclusion Our study suggests that enhanced thrombin generation and prothrombotic plasma fibrin clot phenotype can contribute to increased risk of thrombosis in patients with antithrombin deficiency.

Introduction

Antithrombin, a 58 kDa glycoprotein composed of 432 amino acids in humans encoded by *SERPINC1* (chromosome 1q25.1), is the main endogenous anticoagulant inactivating thrombin, activated factor X (FXa), and to a lesser extent FXIIa, FXIa, and FIXa.[1] Antithrombin has a reactive center that interacts with the active site of coagulation factors and a heparin-binding site, distinct from the reactive center.[1] Following the heparin binding, antithrombin activity is accelerated.[1] Antithrombin is present in blood at a concentration of approximately 0.23 g/L. Its activity ranges from 80 to 120% (mostly 90–110%) in different functional assays and its half-life is approximately 3 days.

Antithrombin deficiency, a disorder first described in 1965, is the strongest congenital thrombophilia, as it associates with early and recurrent onset of venous thromboembolism (VTE).[2,3] A meta-analysis of 35 studies showed that the odds ratio (OR) for the first VTE among individuals with antithrombin deficiency is 14 (95% confidence interval [CI]: 5.5–29) with the annual risk of 1.2% (95% CI: 0.8–1.7).[4]

The prevalence of antithrombin deficiency is estimated from 0.02% to 0.2% (1/5,000–1/500 individuals), equally common in both sexes.[1] Genetic variants in *SERPINC1* (407 recorded at Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1) may cause type I deficiencies if they reduced protein functional activity and antigen levels, or type II if they only affect the activity of the variant.[5] Type II deficiencies are related to alterations of the reactive site of antithrombin (type IIRS), heparin-binding site (type IIHBS), or pleiotropic effects (type IIPE), involving defects at the carboxy terminal end of antithrombin molecule.[6,7] Up to 5% of patients with antithrombin deficiency are explained by a global N-glycosylation defect characterized by increased hypoglycosylated forms of antithrombin.[7,8] Even a mild antithrombin deficiency significantly increases the risk of thrombosis, as demonstrated by different studies.[9,10]

The prothrombotic role of antithrombin deficiency has been associated with increased thrombin generation of carriers[11,12] even in particular congenital antithrombin deficiencies not detected by functional assays.[13] An impaired anticoagulant activity might also affect the structure of the fibrin clot with prothrombotic consequences. Indeed, the thickness of fiber strands and porosity of the gel increased with increasing antithrombin concentrations in a model with antithrombin-depleted plasma[14] and a patient with congenital type I deficiency (anti-FXa: 56.3%) caused by the heterozygous deletion of nucleotide A in codon 438 in the last exon of *SERPINC1* gene was shown to be associated with 26% decreased fibrin clot permeability and 33% prolonged clot lysis time (CLT) compared with control with a substantial improvement of fibrin clot characteristics after normalization of antithrombin activity.[15] Impaired fibrin properties contribute to so-called prothrombotic fibrin clot phenotype, which has been associated with increased risk of VTE, its recurrence, and complications such as postthrombotic syndrome.[16] A proteomic analysis showed that antithrombin is present within fibrin clot[17] and potentially may influence its properties. We hypothesized that antithrombin deficiency affects fibrin clot structure and function, independently of its impact on thrombin generation, and may help to identify patients at high risk of thrombosis.

Materials and Methods

Patients

We enrolled 148 patients with genetically confirmed antithrombin deficiency (see ►**Supplementary Material**, available in the online version). Patients were recruited between August 2019 and July 2022 in four centers for coagulation disorders, namely two in Poland (Krakow and Lodz), Hungary (Debrecen), and Spain (Murcia). Fifty healthy individuals were recruited in Krakow in the Center for Coagulation Disorders and served as controls. Jagiellonian University Ethical Committee approved the study and all the participants provided their written informed consent in accordance with the Declaration of Helsinki.

Laboratory Investigations

All laboratory investigations were performed in the Center for Coagulation Disorders in Kraków by experienced investigators who were blinded to the sample origin. Venous blood samples were collected (see ►**Supplementary Material**, available in the online version). Antithrombin deficiency was classified as previously described.[8] Subjects treated with direct oral anticoagulants (DOACs; rivaroxaban or apixaban; $n = 29$, 19.6%) or low-molecular-weight heparin ($n = 6$, 4%) were asked to take the last dose of drug 24 hours before the visit in the outpatient clinic. Plasma from patients with DOAC blood levels >30 ng/mL ($n = 7$, 4.7%) was treated with the DOAC-Stop (Haematex Research, Sydney, Australia) prior to assessment.[18] Patients with anti-FXa activity <0.2 IU/mL were included in the final analysis.[19] Levels of plasminogen activator-inhibitor type I (PAI-1) antigen (Hyphen BioMed, Neuville-sur-Oise, France), thrombin-activatable fibrinolysis inhibitor (TAFI; Hyphen BioMed) antigen, prothrombin fragments 1 + 2 (F1 + 2, Siemens Healthcare Diagnostics, Marburg, Germany), and FVIIa-antithrombin complex (FVIIa-AT; Diagnostica Stago, Asnières-sur-Seine, France) were assayed by ELISA. The whole *SERPINC1* (NM_000 488.3) gene was analyzed as previously described.[20]

Fibrin Clot Analysis

Fibrin clot permeability (K_s) was determined as described previously.[21] Briefly, 20 mM $CaCl_2$ and 1 U/mL human thrombin (Merck, Darmstadt, Germany) were added to plasma. Alternatively, tissue factor (TF)-based K_s (final TF concentration, 5 pmol/L; Innovin, Siemens) was assessed, since the choice of a coagulation trigger can affect the results.[19] After K_s measurement, clots were assessed using scanning electron microscopy (SEM).[19]

CLT and density (ΔAbs) were measured as described.[22] Briefly, citrated plasma was mixed with 20 mM $CaCl_2$, 0.5 U/mL thrombin (Merck), 15 µM phospholipid vesicles (Rossix, Mölndal, Sweden), and 18 ng/mL recombinant tissue plasminogen activator (tPA; Actilyse 20 mg, Boerhinger Ingelheim, Germany). In the second assay, clot formation was initiated by 6 pM TF (TF-based CLT).[23] For details, see ►**Supplementary Material** (available in the online version).

Calibrated Automated Thrombogram

Thrombin generation kinetics were measured with the Calibrated Automated Thrombogram (CT) (Thrombinoscope BV, Maastricht, the Netherlands).[24] Briefly, 20 µL of the PPP-Reagent (Diagnostica Stago, Asnières sur Seine Cedex, France) containing approximately 5 pM of recombinant TF, 4 µM of phospholipid vesicles, and 20 µL of FluCa solution (Diagnostica Stago) were added to 80 µL of plasma. For details, see ►**Supplementary Material** (available in the online version).

In Vitro Normalization of Antithrombin Activity

Patient plasma was spiked with the stock of 5 to 10 mg/mL antithrombin purified from human citrated plasma (activity ≥5 units/mg, Merck). Ten microliters of antithrombin stock diluted with Tris buffer (0.01 M Tris, 0.1 M NaCl, pH

7.5) were added to 500 µL of plasma to obtain a final antithrombin activity of 100% or 150%. Antithrombin activity after normalization was determined. The accuracy of adjusting antithrombin activity was approximately 4%. Antithrombin-depleted human plasma (BioMedica Diagnostics, Windsor, Nova Scotia, Canada) reconstituted with different concentrations of antithrombin was used to assess associations between antithrombin level and fibrin clot properties.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation or median with interquartile range. Normality of the data was assessed using the Shapiro–Wilk test. Categorical variables were presented as numbers and percentages and were compared by two-sided Pearson's χ^2 or Fisher's exact test. Differences between two groups were compared using the Student's *t*-test for normally distributed continuous variables and for nonnormally 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. Associations between nonparametric or parametric variables were assessed by Spearman's or Pearson's tests, respectively. Based on available data, at least 62 individuals with type I or type II antithrombin deficiency were required.[15]

The univariable linear regression models were performed to identify associations between fibrin clot properties and demographic, clinical, and laboratory variables (see ►**Supplementary Material**, available in the online version). A *p*-value of <0.05 was considered statistically significant. Statistical analysis was performed using STATISTICA 13 (StatSoft STATISTICA, Poland) and R 4.1.1 (The R Foundation for Statistical Computing, Vienna, Austria, 2021).

Results

Antithrombin Deficiency versus Healthy Control

Antithrombin-deficient patients did not differ from healthy controls with regard to age, sex, and body mass index (BMI; ►**Table 1**). Previous VTE occurred in 25% of patients (►**Table 1**). Antithrombin-deficient patients compared with healthy controls had 8.6% lower fibrinogen, 38.6% lower antithrombin activity, and 23.2% lower antithrombin antigen levels (►**Table 1**). Protein C and protein S levels were similar in antithrombin-deficient patients and controls (►**Table 1**). There was no difference in PAI-1 levels between patients and controls, while TAFI antigen levels were reduced by 19.1% in the former group. F1 + 2 levels were 26.5% higher in patients with antithrombin deficiency than in controls. FVIIa-AT levels tended to be higher in patients with antithrombin deficiency compared with controls (►**Table 1**). Antithrombin-deficient patients had 94% increased endogenous thrombin potential (ETP), 108% higher peak thrombin, and 234% higher velocity index, along with 19% shortened lag time and 27% shortened ttPeak compared with healthy controls (►**Table 1**). There were no differences in start tail time in patients compared with controls (►**Table 1**). Antithrombin deficiency was associated with 18.4% reduced K_s and 34.7% prolonged CLT, with no difference in ΔAbs, as compared with controls (►**Table 1**). Antithrombin-deficient patients with a history of VTE had 27% higher ETP (►**Fig. 1C**) and 10% prolonged CLT (►**Fig. 1D**) compared with patients without VTE. No differences in peak thrombin or K_s were found between patients with prior VTE compared with those without VTE (both $p > 0.05$). After exclusion of VTE patients, differences in fibrin clot properties and thrombin generation potential remained significant between antithrombin-deficient patients and healthy controls (data not shown).

Type I versus Type II Antithrombin Deficiency

There were 65 (43.9%) patients with type I and 83 (56.1%) patients with type II antithrombin deficiency, including 6 (7.2%) patients with type IIRS, 73 (88%) with type IIHBS, and 4 (4.8%) patients with type IIPE. There were 109 (73.6%) carriers of missense mutations in the *SERPINC1* gene, such as antithrombin Budapest 3 (p.Leu131Phe; $n = 27$, including two homozygous subjects), Toyama (p.Arg79Cys; $n = 17$), Basel (p.Pro73Leu; $n = 15$), c.1157T > C (p.Ile386Thr; $n = 9$), Cambridge II (p.Ala416Ser; $n = 5$), or Padua (p.Arg79His; $n = 3$). Other identified *SERPINC1* genetic variants were single-nucleotide variants in an intronic region on aberrant pre-mRNA (c.1154–14G > A) ($n = 13$), nonsense mutations ($n = 3$), frameshift mutations ($n = 12$), structural deletions and duplications ($n = 5$), intronic deletions ($n = 2$), and intronic SVA retrotransposon insertion ($n = 1$). In three cases, antithrombin deficiency was caused by a congenital disorder of glycosylation.

A comparison of antithrombin activity and antigen levels, along with fibrin clot properties among patients with various *SERPINC1* genetic variants, is presented in ►**Fig. 2**.

Patients with type I and type II antithrombin deficiency did not differ with regard to age, gender, BMI, and the incidence of previous VTE, including unprovoked events (►**Table 1**). Antithrombin activity and antithrombin antigen levels were reduced by 22.5% and by 40% in type I compared with type II antithrombin deficiency, respectively (►**Table 1**). We found no differences in plasma fibrinogen, PAI-1, FVIIa-AT, and F1 + 2 between subjects with I or II antithrombin deficiency (►**Table 1**). TAFI activity was 10.2% higher in patients with type I compared with type II antithrombin deficiency (►**Table 1**). Among the CT parameters, solely ETP was 30% higher in patients with type I compared with type II antithrombin deficiency (►**Table 1**).

Patients with type I compared with type II antithrombin deficiency had 8.4% reduced K_s (►**Fig. 1A**) and 18% prolonged CLT (►**Fig. 1B**), while no difference in ΔAbs was found (►**Table 1**). Similar results were obtained for TF-based K_s (−8%) and TF-based CLT (−15% in type I vs. II antithrombin deficiency; data not shown). Of note, both TF-based K_s and TF-based CLT correlated with K_s (rho = 0.74, $p < 0.01$) and CLT (rho = 0.68, $p < 0.0001$).

The sub-analysis involving antithrombin-deficient patients after exclusion of subjects with previous VTE revealed that both individuals with type I and type II antithrombin

Table 1 Characteristics of type I and type II antithrombin-deficient patients

Variable	Antithrombin deficiency ($n = 148$)	Healthy control ($n = 50$)	*p*-Value	Type I antithrombin deficiency ($n = 65$, 43.9%)	Type II antithrombin deficiency ($n = 83$, 56.1%)	*p*-Value
Age, y	38 [32–50]	39 [34–43]	0.80	38 [29–50]	39 [33–49]	0.44
Female, *n* (%)	103 (69.6)	35 (70)	0.96	43 (66.2)	60 (72.3)	0.42
BMI, kg/m^2	24.4 ± 4.2	25.1 ± 4.2	0.95	24.4 ± 4.3	24.3 ± 4.2	0.97
Previous VTE, *n* (%)	38 (25)	0	<0.0001	19 (29.2)	19 (22.9)	0.38
Unprovoked VTE, *n* (%)	17 (11.2)	0	0.0077	11 (16.9)	6 (7.2)	0.072
Fibrinogen, g/L	2.66 [2.32–3.11]	2.91 [2.53–3.38]	0.0082	2.73 [2.34–3.13]	2.63 [2.32–3.05]	0.58
Antithrombin activity, %	62 [50–75]	101 [94–110]	<0.0001	55 [50–63]	71 [50–80]	0.0001
Antithrombin antigen, g/L	0.192 [0.144–0.235]	0.250 [0.226–0.280]	<0.0001	0.138 [0.117–0.156]	0.230 [0.212–0.250]	<0.0001
Protein C, %	111 [97–136]	109 [98–119]	0.88	110 [95–132]	109 [96–137]	0.87
Protein S, %	97 [91–110]	102 [92–115]	0.69	99 [92–111]	96 [90–109]	0.79
PAI-1, ng/mL	12.0 [8.8–19.3]	11.9 [10.0–14.3]	0.74	12.1 [7.3–19.5]	12.0 [9.1–19.3]	0.53
TAFI, %	72.0 ± 17.6	89.0 ± 11.0	<0.0001	75.9 ± 20.3	68.9 ± 14.6	0.018
FVIIa-AT, pM	135 [107–168]	124 [103–158]	0.062	135 [108–168]	135 [106–168]	0.74
F1 + 2 prothrombin fragments, pM	196 [146–249]	155 [124–191]	0.0029	197 [154–274]	194 [142–246]	0.27
Lag time, min	2.82 [2.55–3.04]	3.47 [2.94–3.68]	<0.0001	3.00 [2.37–3.00]	2.67 [2.55–3.13]	0.93
ETP, nM × min	2,225 [1,772–2,782]	1,148 [1,069–1,329]	<0.0001	2,582 [1,994–2,999]	1,992 [1,691–2,493]	0.0003
ttPeak, min	5.90 [5.33–6.67]	8.12 [7.09–9.26]	<0.0001	6.00 [5.00–6.94]	5.72 [5.33–6.67]	0.85
Peak thrombin, nM	324 [254–388]	156 [135–208]	<0.0001	328 [245–394]	324 [257–378]	0.73
Start tail, min	23.8 [21.3–27.3]	23.2 [21.7–25.2]	0.20	24.3 [18.3–28.0]	23.4 [21.9–27.2]	0.86
Velocity index, nM/min	117 [82–146]	35 [25–52]	<0.0001	119 [82–159]	115 [77–137]	0.22
K_s, $\times 10^{-9}$ cm^2	6.37 [5.22–7.58]	7.81 [7.34–8.11]	<0.0001	5.92 [4.89–6.96]	6.46 [5.55–8.18]	0.0083
CLT, min	128 [112–145]	95 [79–103]	<0.0001	138 [122–149]	117 [107–132]	<0.0001
ΔAbs, AU	0.274 [0.220–0.316]	0.290 [0.238–0.340]	0.18	0.269 [0.179–0.300]	0.280 [0.233–0.330]	0.067

Abbreviations: ΔAbs, absorbance of the fibrin clot; BMI, body mass index; CLT, clot lysis time; ETP, endogenous thrombin potential; FVIIa-AT, activated factor VII-antithrombin complex; K_s, fibrin clot permeability; PAI-1, plasminogen activator inhibitor type 1; TAFI, thrombin-activatable fibrinolysis inhibitor; VTE, venous thromboembolism.

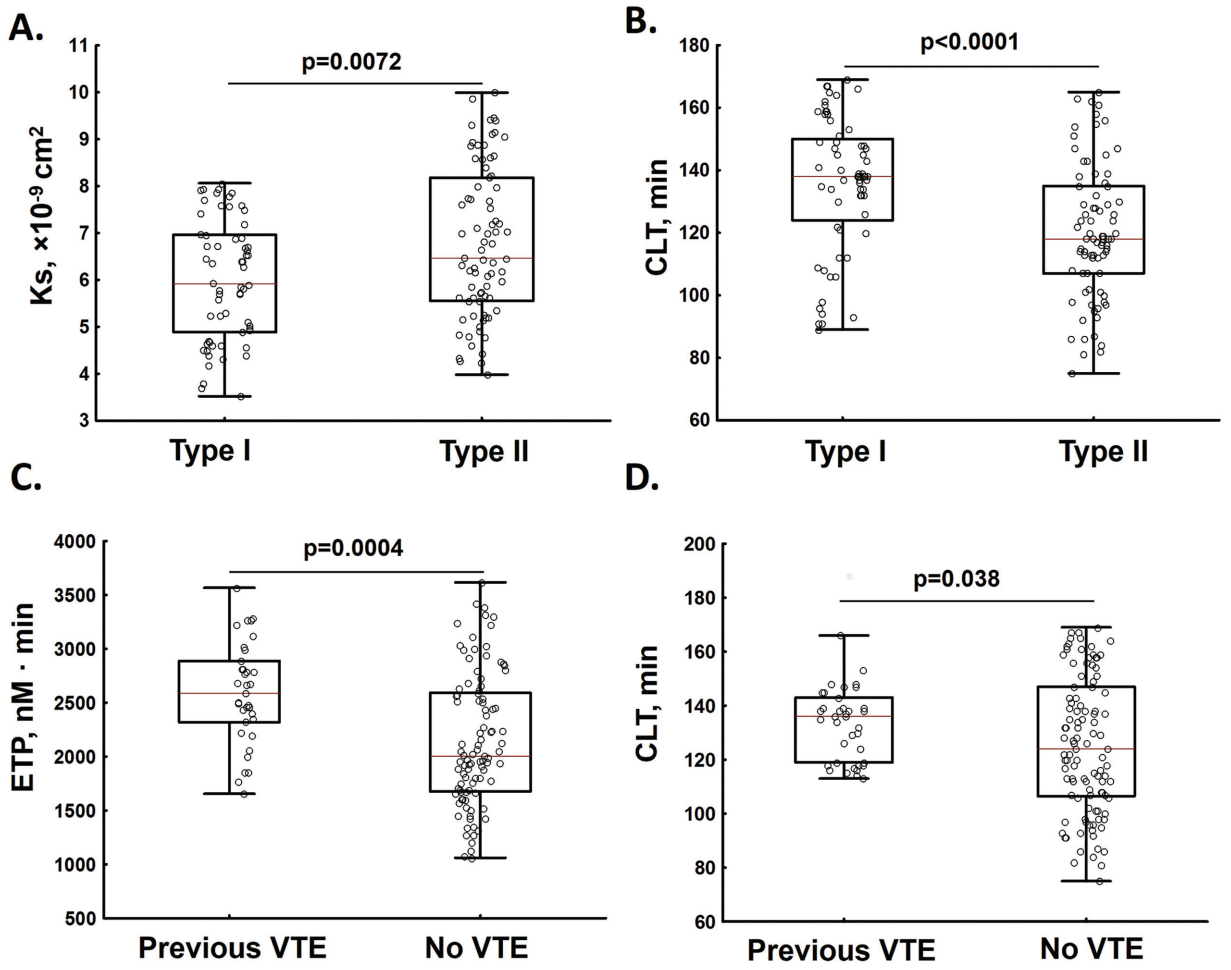

Fig. 1 Fibrin clot permeability (K_s) (**A**) and clot lysis time (CLT) (**B**) in patients with type I compared with type II antithrombin deficiency, and endogenous thrombin potential (ETP) (**C**) and CLT (**D**) in antithrombin-deficient patients with previous VTE compared with those without VTE history. VTE, venous thromboembolism.

deficiency had increased thrombin generation and unfavorably altered fibrin clot properties compared with controls (►**Table 2**). After excluding VTE patients, TAFI activity was similar in type I and type II antithrombin deficiency ($p > 0.05$). ETP (+33.4%), peak thrombin (+10.5%), and velocity index (+17.6%) were higher along with prolonged CLT (+18.1) observed in type I compared with type II antithrombin deficiency patients with no VTE history (►**Table 2**).

Fibrin Clot Morphology

Patients with antithrombin deficiency formed 34.5% thinner fibrin fibers as compared with controls (76 [67–80] nm vs. 116 [105–141] nm, $p < 0.0001$). SEM analysis revealed no difference in fibrin fiber diameter between individuals with type I and type II antithrombin deficiency (75 [62–80] vs. 77 [71–85] nm, $p = 0.095$; ►**Fig. 3**). Of note, 27.5% of analyzed clots were characterized by numerous small fibrin fibers or fiber ends throughout the clot (►**Fig. 4**), which were observed mainly in patients with increased F1 + 2 levels (285 [214–312] vs. 187 [156–214] pM, $p = 0.013$), regardless of the type of antithrombin deficiency. Abnormal fibrin fibers were not observed in healthy subjects.

Associations of Fibrin Clot Properties

K_s and CLT were weakly associated with antithrombin activity (rho = 0.20, $p = 0.021$ and rho = − 0.19, $p = 0.019$, respectively) and antigen levels (rho = 0.18, $p = 0.032$ and rho = − 0.31, $p = 0.0002$, respectively). CLT was moderately associated with PAI-1 levels (rho = 0.38, $p < 0.0001$) and TAFI activity (rho = 0.45, $p < 0.0001$) in antithrombin-deficient patients. There were no associations between fibrin clot properties and FVIIa-AT, F1 + 2, or thrombin generation parameters (data not shown), except an inverse association between fibrin diameter and F1 + 2 (rho = − 0.57, $p = 0.00012$). ETP but not peak thrombin was negatively associated with antithrombin antigen (rho = − 0.30, $p = 0.0005$).

The linear regression analysis adjusted for age, sex, previous VTE, and fibrinogen levels showed that a lower antithrombin antigen level (per 1 g/L decrease; β = − 6.1, 95% CI: −1.7 to −10.5) was independently associated with reduced K_s, while prolonged CLT was associated with lower antithrombin antigen (per 1 g/L decrease; β = 69.6, 95% CI: 9.6–129.7) and activity (per 10% decrease; β = 2.4, 95% CI: 0.3–4.5) along with higher PAI-1 (per 10% increase; β = 12.1, 95% CI: 7.7–16.5) and TAFI levels (per 10% increase; β = 3.8, 95% CI: 1.9–5.7).

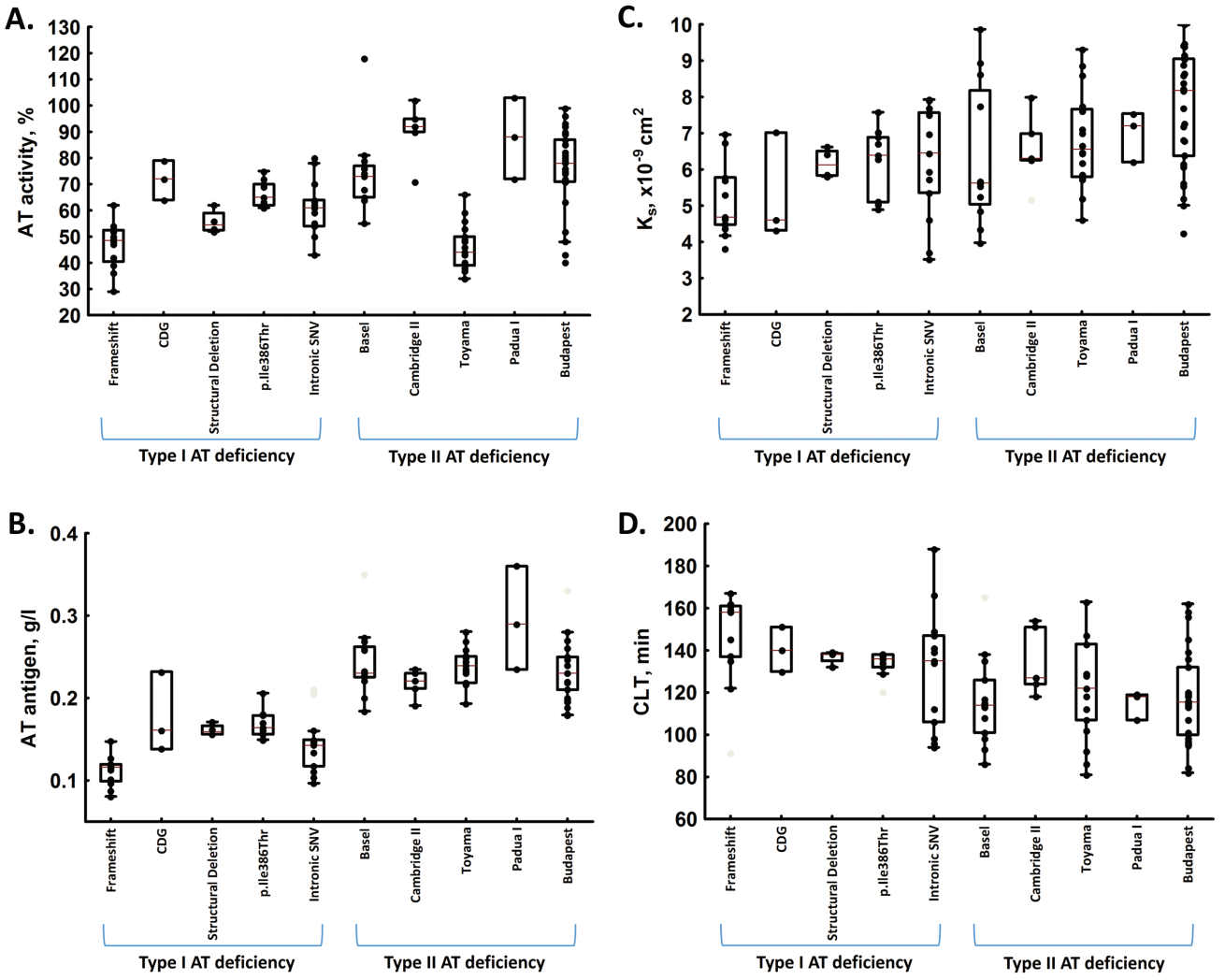

Fig. 2 Antithrombin (AT) activity ($p < 0.0001$ for ANOVA) (**A**), AT antigen (p = 0.99 for ANOVA) (**B**), fibrin clot permeability (K_s, $p = 0.0039$ for ANOVA) (**C**), and clot lysis time (CLT; p = 0.001 for ANOVA) (**D**) in antithrombin-deficient patients with different types of mutation in the SERPINC1 gene.

In Vitro Antithrombin Normalization

Normalization of antithrombin activity to obtain 100% in plasma of 50 patients with type I ($n = 25$; 50%) or type II antithrombin deficiency (mean antithrombin activity: 61 ± 12%) was associated with 39% reduced ETP (2,455 [2,457–2,875] vs. 1,497 [1,264–1,676] nMxmin, $p < 0.0001$), 18% reduced peak thrombin (304 [246–399] vs. 250 [203–314] nM, $p < 0.0001$), 8% increased K_s (►**Fig. 5A**), and 12% shortened CLT (►**Fig. 5B**). Only in 6 (12%) patients we found improvement of both K_s and CLT >5%, in 20 subjects (40%) we observed CLT shortening of >5%, and in 13 (26%) individuals K_s increased >5%.

Supplementation of antithrombin-deficient patients' plasma with antithrombin resulted in reduced ETP (►**Fig. 6A**) and reduced peak thrombin (►**Fig. 6B**) in a dose-dependent manner. No differences were found in lag time and ttPeak after addition of exogenous antithrombin ($p > 0.05$ for both). The dose-dependent effects related to antithrombin addition to plasma obtained from antithrombin-deficient patients were not observed for K_s or CLT, independently of thrombin or TF used as a coagulation activator (data not shown). However, an increase of antithrombin activity in the range from 50 to 100% in normal citrated human plasma immunodepleted of antithrombin was related to 84% higher K_s (►**Fig. 7A**) and CLT shortened by 12% (►**Fig. 7B**).

Discussion

This study showed for the first time that antithrombin deficiency contributes to increased thrombin generation and prothrombotic fibrin clot phenotype. Moreover, clots from subjects with type I compared with type II antithrombin deficiency were denser and more resistant to lysis in association with lower antithrombin activity and level. The genetic background seemed to influence the fibrin clot properties, but numbers were small and there was a large variability between patients within each genetic category, depending on antithrombin activity and fibrinogen levels. Importantly, in contrast to antithrombin-depleted normal plasma, we found only a slight increase in K_s after antithrombin activity normalization in antithrombin-deficient patients. This observation suggests that the effects of

Table 2 Characteristics of type I and type II antithrombin-deficient patients without previous venous thromboembolism (VTE)

Variable	Type I antithrombin deficiency without previous VTE ($n = 46$)	Type II antithrombin deficiency without previous VTE ($n = 64$)	Healthy control ($n = 50$)
Age, y	37 [29–44]	41 [34–49]	39 [34–43]
Female, *n* (%)	33 (71.7)	51 (79.7)	35 (70)
Fibrinogen, g/L	2.61 [2.09–3.04][a]	2.66 [2.35–3.01][a]	2.91 [2.53–3.38]
Antithrombin activity, %	53 [43–61][a]	64 [47–78][a,b]	101 [94–110]
Antithrombin antigen, g/L	0.125 [0.112–0.146][a]	0.230 [0.214–0.250][a,b]	0.250 [0.226–0.280]
PAI-1, ng/mL	14.0 [8.1–20.3]	12.2 [9.9–21.2]	11.9 [10.0–14.3]
TAFI, %	70.9 ± 17.6[a]	70.4 ± 12.4[a]	89.0 ± 11.0
FVIIa-AT, pM	134 [110–171]	141 [106–172]	124 [103–158]
F1 + 2 prothrombin fragments, pM	198 [160–286][a]	198 [145–237][a]	155 [124–191]
Lag time, min	3.00 [2.33–3.00][a]	2.67 [2.55–3.08][a]	3.47 [2.94–3.68]
ETP, nM × min	2,575 [1,920–3,001][a]	1,931 [1,530–2,234][a,b]	1,148 [1,069–1,329]
ttPeak, min	6.00 [5.00–6.90][a]	5.88 [5.33–6.67][a]	8.12 [7.09–9.26]
Peak thrombin, nM	358 [293–420][a]	324 [255–369][a,b]	156 [135–208]
Start tail, min	24.1 [18.3–27.0]	23.5 [21.7–27.1]	23.2 [21.7–25.2]
Velocity index, nM/min	120 [82–160][a]	102 [72–130][a,b]	35 [25–52]
K_s, $\times 10^{-9}$ cm^2	5.78 [4.64–6.98][a]	6.23 [5.22–7.87][a]	7.81 [7.34–8.11]
CLT, min	137 [112–158][a]	116 [100–135][a,b]	95 [79–103]
ΔAbs, AU	0.268 [0.167–0.300][a]	0.277 [0.234–0.322]	0.290 [0.238–0.340]

Abbreviations: ΔAbs, absorbance of the fibrin clot; BMI, body mass index; CLT, clot lysis time; ETP, endogenous thrombin potential; FVIIa-AT, activated factor VII-antithrombin complex; K_s, fibrin clot permeability; PAI-1, plasminogen activator inhibitor type 1; TAFI, thrombin-activatable fibrinolysis inhibitor.

[a] $p < 0.05$ compared with controls.

[b] $p < 0.05$ for comparison of type I versus type II antithrombin deficiency.

antithrombin deficiency on fibrin clot properties could be, at least in part, mediated by other factors than low antithrombin activity, such as posttranslational modifications of the fibrinogen molecule.

Previous data indicate that healthy women heterozygous for FV Leiden genetic variant formed fibrin clots more resistant to fibrinolysis compared with noncarriers,[25] which can be associated with a delay in tPA-induced clot lysis in FV Leiden carriers.[26] VTE patients with prothrombin G20210A genetic variant formed denser fibrin clots with impaired susceptibility to fibrinolysis compared with prothrombin G20210A noncarriers.[27] Foley et al[28] reported impaired dynamics of thrombin–antithrombin complex formation, which contributed to increased clot density, enhanced consumption of factor XIII, and fibrin resistance to fibrinolysis in patients with protein C deficiency compared with healthy controls. To the best of our knowledge, reports on plasma fibrin clot properties in antithrombin-deficient patients are sparse. Celinska-Lowenhoff et al[15] showed approximately 26% decreased K_s and 33% prolonged CLT in the patient with type I antithrombin deficiency (antithrombin Krakow) compared with control, suggesting a presence of prothrombotic mechanisms associated with antithrombin deficiency in patients with a history of thrombosis. In a recent study, Smith et al[29] showed in a cohort of 14 young subjects with type I and II antithrombin deficiency without anticoagulant therapy that increased ETP is able to identify individuals at highest risk for thrombosis with no difference between symptomatic and asymptomatic family members. Importantly, D-dimer concentrations were moderately increased in about half of the individuals, while thrombin–antithrombin levels were elevated only in a few subjects.[29] In the current study, we found higher differences in ETP than in peak thrombin between patients with type I compared with type II antithrombin deficiency. Dielis et al[30] reported that FXa-based antithrombin activity and prothrombin levels were the most important determinants of ETP and to lower extent of peak thrombin in healthy individuals, especially at higher levels of TF used as clotting activator (13.6 pM). This observation may explain our findings that antithrombin normalization markedly reduced ETP and to lower extent peak thrombin in the dose-dependent manner. Normalization of antithrombin activity improved also K_s and CLT but all parameters did not reach the values observed in controls. Moreover, antithrombin normalization did not prolong the lag time or ttPeak, which indicates another mechanism involved in regulation of the initiation phase of thrombin generation in antithrombin-deficient patients. Both TF pathway inhibitor (TFPI) and antithrombin are the principal stoichiometric inhibitors of

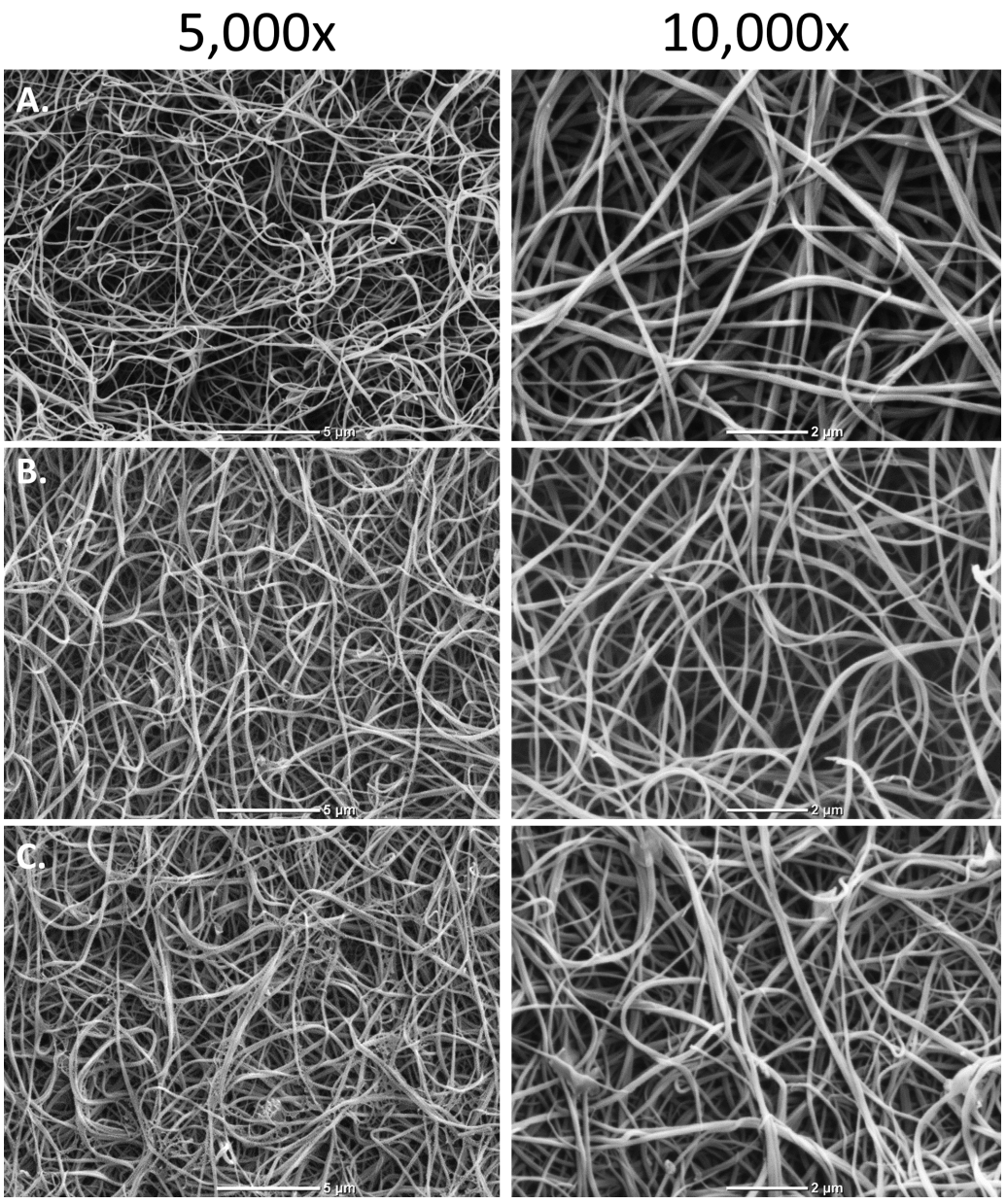

Fig. 3 Representative scanning electron microscopy micrographs of fibrin clot prepared from citrated plasma obtained from healthy control (**A**) and patients with type I (**B**) or type II (**C**) antithrombin deficiency with similar fibrinogen levels (2.79–2.83). Magnification: 5,000× and 10,000 ×; scale bar: 5 and 2 µm.

thrombin generation.[5] TFPI is a regulator of the initiation phase, while antithrombin attenuates thrombin activity during the propagation phase.[31] TFPI is also a reversible inhibitor of FXa during the propagation phase of thrombin generation.[32] We hypothesize that antithrombin deficiency influences the balance between TF-FVIIa and FXa inhibition by TFPI. It has been shown that approximately 5% of thrombin generated during the initiation phase of thrombin generation is enough to form the clot and the rate of generated thrombin during the early phase of coagulation activation determines the final fibrin structure.[31–33] In the current study antithrombin-deficient patients had slightly higher F1 + 2 levels (mostly within the normal range) as in vivo markers of thrombin generation compared with controls, along with very high potential for thrombin generation in vitro. It has been reported that fibrin clots formed at higher thrombin concentrations are composed of thinner and tightly packed fibrin fibers,[34] which are usually less susceptible for fibrinolysis. Formation of thinner fibers is related with a slower rate of fibrinopeptide B release.[31,33] SEM analysis confirmed these observations by showing abnormal fibrin structure with additional small fibrin fibers, which were associated with increased F1 + 2 levels in antithrombin-deficient patients. Similar fibrin network abnormalities have been reported in clots formed from a recombinant α390 fibrinogen variant without the C-terminal domain.[35] The lack of C-terminal domain, which αC-region normally interacts with fibrinopeptide B, prevented lateral aggregation of protofibrils, resulting in formation of thinner fibrin fibers. We hypothesize that a higher rate of thrombin generation can impair normal fibrin polymerization and longitudinal fiber growth. We found

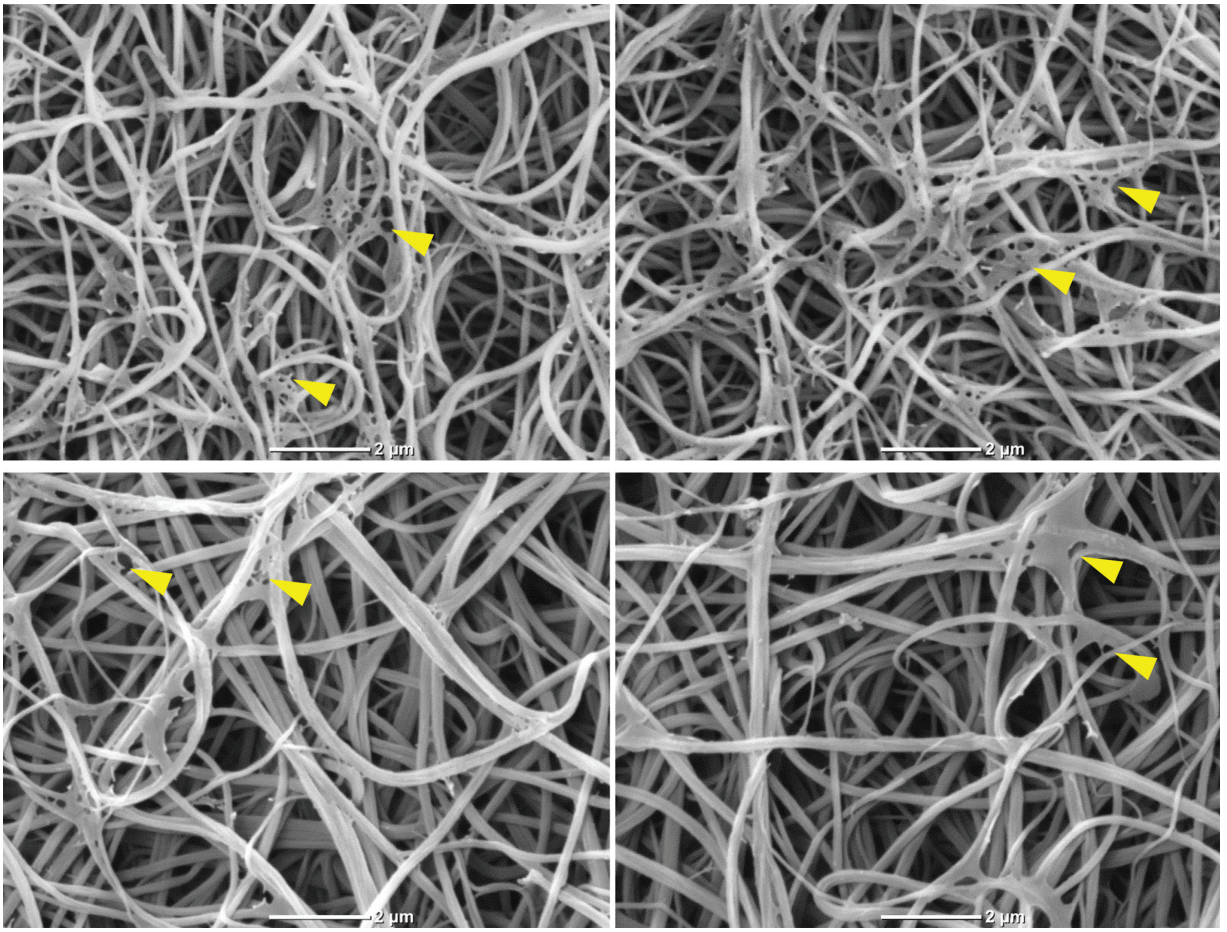

Fig. 4 Abnormal fibrin fibers present in clots of a subset of antithrombin-deficient patients. Magnification: 10,000 ×, scale bar: 2 µm.

no difference in fibrin fiber diameter between patients with type I and II antithrombin deficiency; however, this observation is in line with similar ΔAbs and levels of F1 + 2 in both groups. Further studies are needed to investigate the impact of fibrinopeptide B release at high thrombin amounts on fibrin structure in antithrombin-deficient patients.

In the current study, previous VTE in antithrombin-deficient patients was associated with higher ETP and prolonged CLT compared with individuals without VTE. Increased ETP was independently associated with a history of VTE (OR: 1.04, 95% CI: 1.02–1.08 per 1% increase).[36] Prothrombotic fibrin clot phenotype was reported in patients with a history of first-ever VTE,[37] while prolonged CLT in women was associated with recurrent VTE in a prospective study.[38] Our observation suggests that impaired ETP and CLT can be persistent features of patients with inherited thrombophilia and previous VTE, who are at high risk of recurrent events and may require additional thromboprophylaxis. A personalized anticoagulant therapy based on clinical risk factors and fibrin clot properties could be beneficial to prevent VTE in this group of patients.

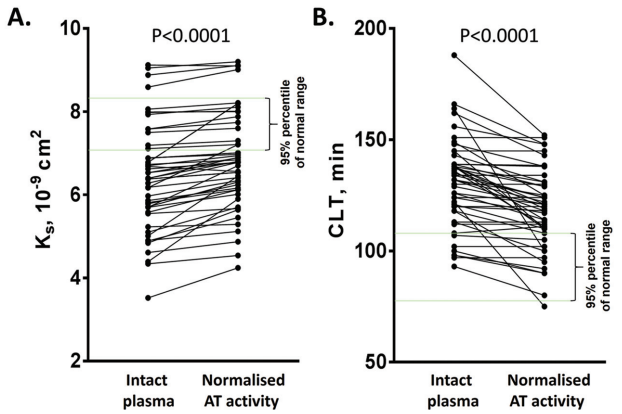

Fig. 5 Fibrin clot permeability (K_s) (**A**) and clot lysis time (CLT) (**B**) after normalization of antithrombin (AT) activity. Data compared using the Wilcoxon signed-rank test.

This study has several limitations. First, the sample size was limited. Moreover, individuals with transient antithrombin deficiency or with acquired antithrombin deficiency were not assessed. Third, all experiments in patients taking DOACs were done after using DOAC-Stop, which has been shown to provide appropriate results of antithrombin deficiency screening.[18] However, the DOAC-Stop treatment or residual FXa activity might interfere with some of the assays performed in this study.

This study indicates that antithrombin deficiency is associated with enhanced thrombin generation and unfavorably altered fibrin clot structure and function, which is at least in part determined by the *SERPINC1* genetic background.

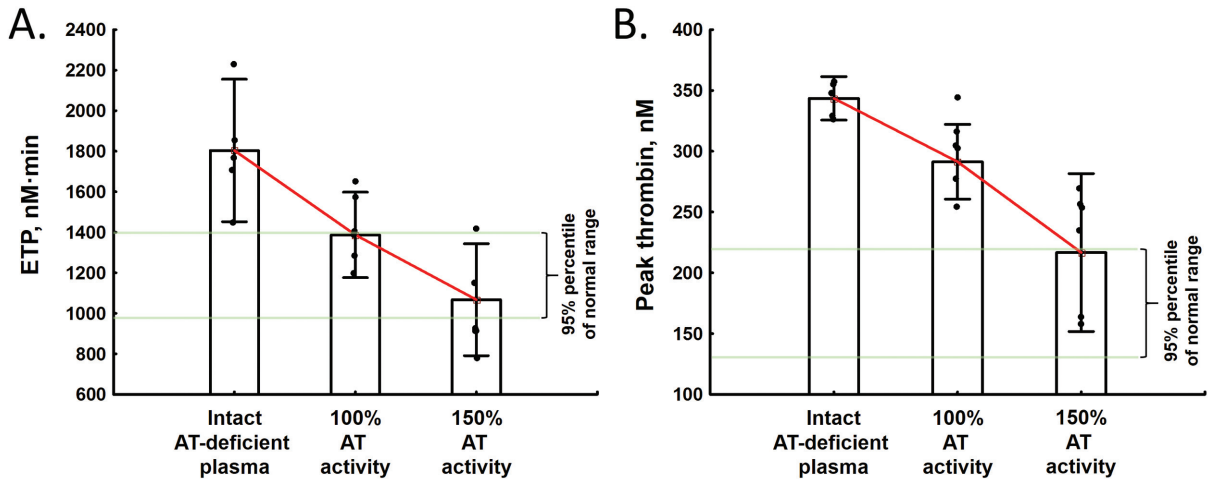

Fig. 6 A dose-dependent effect of antithrombin addition on (**A**) endogenous thrombin potential (ETP) and (**B**) peak thrombin (both *p* for trend <0.001).

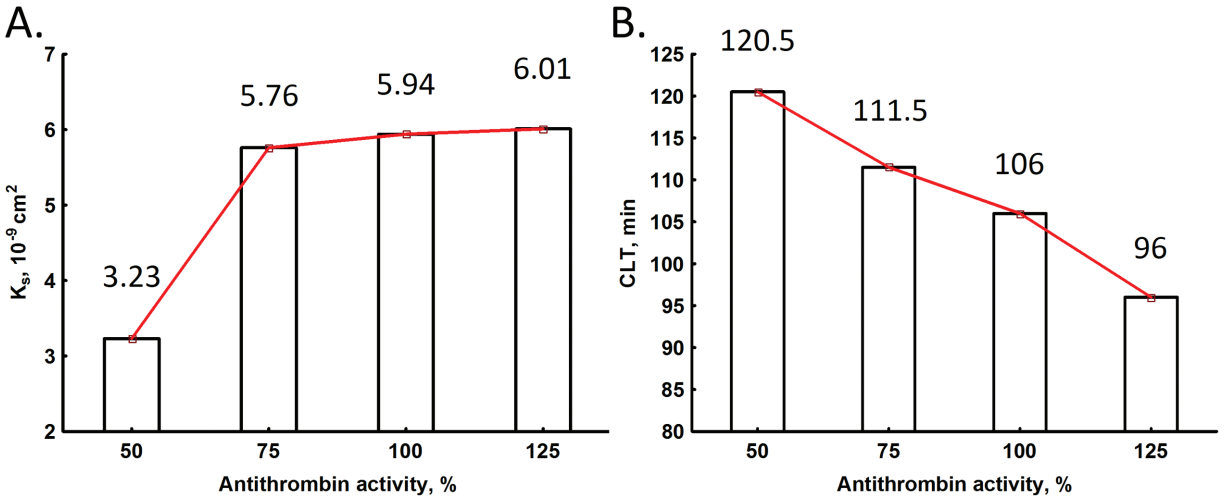

Fig. 7 A dose-dependent effect on K_s (**A**) and CLT (**B**) after antithrombin addition to commercial normal citrated human plasma immunodepleted of antithrombin (fibrinogen concentration: 2.82 g/L) (both *p* for trend <0.001). Data are presented as a mean for three experiments.

What is known about this topic?

- Antithrombin (AT) deficiency contributes to venous thromboembolism (VTE).
- Antithrombin deficiency has been associated with increased thrombin generation.
- Prothrombotic fibrin clot phenotype has been associated with increased risk of VTE, its recurrence, and complications.

What does this paper add?

- Prothrombotic plasma fibrin clot phenotype is associated with AT deficiency.
- More prothrombotic clot features were related to type I AT deficiency and VTE history.
- Prothrombotic plasma fibrin clot phenotype may contribute to increased risk of thrombosis in AT deficiency.

Authors' Contribution
Conception and design of the study: M. Z.; data collection, analysis, and interpretation: J. N., J. C., M. E. M.-B., C. B.-P., Z. Ba., Z. Be., J. T., M. W., A. K., and A. U.; drafting the article: J. N. and M. Z.; revising the article: J. C., M. E. M.-B., C. B.-P., Z. Ba., Z. Be., J. T., M. W., A. K., and A. U.

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Conflict of Interest
None declared.

References

1 Patnaik MM, Moll S. Inherited antithrombin deficiency: a review. Haemophilia 2008;14(06):1229–1239
2 Limperger V, Franke A, Kenet G, et al. Clinical and laboratory characteristics of paediatric and adolescent index cases with venous thromboembolism and antithrombin deficiency. An observational multicentre cohort study. Thromb Haemost 2014;112 (03):478–485
3 de la Morena-Barrio B, Orlando C, de la Morena-Barrio ME, Vicente V, Jochmans K, Corral J. Incidence and features of thrombosis in children with inherited antithrombin deficiency. Haematologica 2019;104(12):2512–2518
4 Croles FN, Borjas-Howard J, Nasserinejad K, Leebeek FWG, Meijer K. Risk of venous thrombosis in antithrombin deficiency: a systematic review and Bayesian meta-analysis. Semin Thromb Hemost 2018;44(04):315–326
5 Lane DA, Bayston T, Olds RJ, et al; For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Antithrombin mutation database: 2nd (1997) update. Thromb Haemost 1997;77(01):197–211
6 Picard V, Nowak-Göttl U, Biron-Andreani C, et al. Molecular bases of antithrombin deficiency: twenty-two novel mutations in the antithrombin gene. Hum Mutat 2006;27(06):600
7 de la Morena-Barrio ME, Martínez-Martínez I, de Cos C, et al. Hypoglycosylation is a common finding in antithrombin deficiency in the absence of a SERPINC1 gene defect. J Thromb Haemost 2016;14(08):1549–1560
8 Wypasek E, Corral J, Alhenc-Gelas M, et al. Genetic characterization of antithrombin, protein C, and protein S deficiencies in Polish patients. Pol Arch Intern Med 2017;127(7-8):512–523
9 Bucciarelli P, Passamonti SM, Biguzzi E, et al. Low borderline plasma levels of antithrombin, protein C and protein S are risk factors for venous thromboembolism. J Thromb Haemost 2012;10 (09):1783–1791
10 Sokol J, Timp JF, le Cessie S, et al. Mild antithrombin deficiency and risk of recurrent venous thromboembolism: results from the MEGA follow-up study. J Thromb Haemost 2018;16(04):680–688
11 Boyer C, Wolf M, Lavergne JM, Larrieu MJ. Thrombin generation and formation of thrombin-antithrombin III complexes in congenital antithrombin III deficiency. Thromb Res 1980;20(02): 207–218
12 Alhenc-Gelas M, Canonico M, Picard V. Influence of natural SERPINC1 mutations on ex vivo thrombin generation. J Thromb Haemost 2010;8(04):845–848
13 de la Morena-Barrio ME, Suchon P, Jacobsen EM, et al. Two SERPINC1 variants affecting N-glycosylation of Asn224 cause severe thrombophilia not detected by functional assays. Blood 2022;140(02):140–151
14 Elgue G, Sanchez J, Fatah K, Olsson P, Blombäck B. The effect of plasma antithrombin concentration on thrombin generation and fibrin gel structure. Thromb Res 1994;75(02):203–212
15 Celinska-Lowenhoff M, Iwaniec T, Alhenc-Gelas M, Musial J, Undas A. Arterial and venous thrombosis and prothrombotic fibrin clot phenotype in a Polish family with type 1 antithrombin deficiency (antithrombin Krakow). Thromb Haemost 2011;106 (02):379–381
16 Undas A. Fibrin clot properties and their modulation in thrombotic disorders. Thromb Haemost 2014;112(01):32–42
17 Ząbczyk M, Stachowicz A, Natorska J, Olszanecki R, Wiśniewski JR, Undas A. Plasma fibrin clot proteomics in healthy subjects: relation to clot permeability and lysis time. J Proteomics 2019;208:103487
18 Ząbczyk M, Natorska J, Kopytek M, Malinowski KP, Undas A. The effect of direct oral anticoagulants on antithrombin activity testing is abolished by DOAC-Stop in venous thromboembolism patients. Arch Pathol Lab Med 2021;145(01):99–104
19 Yeromonahos C, Marlu R, Polack B, Caton F. Antithrombin-independent effects of heparins on fibrin clot nanostructure. Arterioscler Thromb Vasc Biol 2012;32(05):1320–1324
20 de la Morena-Barrio M, Sandoval E, Llamas P, et al. High levels of latent antithrombin in plasma from patients with antithrombin deficiency. Thromb Haemost 2017;117(05):880–888
21 Mills JD, Ariëns RA, Mansfield MW, Grant PJ. Altered fibrin clot structure in the healthy relatives of patients with premature coronary artery disease. Circulation 2002;106(15):1938–1942
22 Pieters M, Philippou H, Undas A, de Lange Z, Rijken DC, Mutch NJSubcommittee on Factor XIII and Fibrinogen, and the Subcommittee on Fibrinolysis. An international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: communication from the SSC of the ISTH. J Thromb Haemost 2018;16(05):1007–1012
23 Lisman T, Leebeek FWG, Mosnier LO, et al. Thrombin-activatable fibrinolysis inhibitor deficiency in cirrhosis is not associated with increased plasma fibrinolysis. Gastroenterology 2001;121(01): 131–139
24 Hemker HC. Thrombin generation in a reconstituted system: a comment. Thromb Haemost 2002;87(03):551–554
25 Krzek M, Ciesla-Dul M, Zabczyk M, Undas A. Fibrin clot properties in women heterozygous for factor V Leiden mutation: effects of oral contraceptives. Thromb Res 2012;130(04): e216–e221
26 Koncz Z, Bagoly Z, Haramura G, Mezei ZA, Muszbek L. Thrombomodulin-dependent effect of factor V Leiden mutation on the cross-linking of α2-plasmin inhibitor to fibrin and its consequences on fibrinolysis. Thromb Res 2012;130(03):528–534
27 Janion-Sadowska A, Natorska J, Siudut J, Ząbczyk M, Stanisz A, Undas A. Plasma fibrin clot properties in the G20210A prothrombin mutation carriers following venous thromboembolism: the effect of rivaroxaban. Thromb Haemost 2017;117(09): 1739–1749
28 Foley JH, Ferris L, Brummel-Ziedins KE. Characteristics of fibrin formation and clot stability in individuals with congenital type IIb protein C deficiency. Thromb Res 2012;129(04): e142–e146
29 Smith N, Warren BB, Smith J, et al. Antithrombin deficiency: a pediatric disorder. Thromb Res 2021;202:45–51
30 Dielis AW, Castoldi E, Spronk HM, et al. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. J Thromb Haemost 2008;6(01): 125–131
31 Mann KG. Thrombin formation. Chest 2003;124(03):4S–10S
32 Lakshmanan HHS, Estonilo A, Reitsma SE, et al. Revised model of the tissue factor pathway of thrombin generation: role of the feedback activation of FXI. J Thromb Haemost 2022;20(06): 1350–1363
33 Mann KG, Brummel K, Butenas S. What is all that thrombin for? J Thromb Haemost 2003;1(07):1504–1514
34 Wolberg AS. Thrombin generation and fibrin clot structure. Blood Rev 2007;21(03):131–142
35 McPherson HR, Duval C, Baker SR, et al. Fibrinogen αC-subregions critically contribute blood clot fibre growth, mechanical stability, and resistance to fibrinolysis. eLife 2021;10:e68761
36 Wichers IM, Tanck MW, Meijers JC, et al. Assessment of coagulation and fibrinolysis in families with unexplained thrombophilia. Thromb Haemost 2009;101(03):465–470
37 Undas A, Zawilska K, Ciesla-Dul M, et al. Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. Blood 2009;114(19):4272–4278
38 Traby L, Kollars M, Eischer L, Eichinger S, Kyrle PA. Prediction of recurrent venous thromboembolism by clot lysis time: a prospective cohort study. PLoS One 2012;7(12):e51447