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Investigation on the role of α_2-plasmin inhibitor in thrombotic diseases

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INVESTIGATION ON THE ROLE OF α_2-PLASMIN INHIBITOR IN THROMBOTIC DISEASES

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1. INTRODUCTION

Thrombotic diseases are playing the leading role in both mortality and morbidity. The vascular occlusion by thrombi may affect both arterial and venous circulation.

The alpha2-plasmin inhibitor is the primary inhibitor of plasmin, it may play an important role in the formation and maintenance of thrombosis. In parallel with the formation of the fibrin network, activated Factor XIII (FXIIIa) cross-links α_2-PI to the α-chain of fibrin, thus it protects the clot against degradation by plasmin. In the circulation α_2-PI is cleaved both N- and C-terminally. The primary substrate of FXIIIa is the C-terminally intact α_2-PI form, and it binds the N-terminally cleaved form to fibrin more rapidly.

It has been shown in previous studies, that the resistance of the newly formed thrombus to early fibrinolysis depends largely on the incorporation of α_2-PI into the fibrin clot. Despite the importance of this regulatory step and its potential effect on a wide range of thrombotic clinical events, surprisingly little is known about how α_2-PI binds to fibrin under different conditions. Based on results from various animal experiments, it has been found that there is an association between the outcome of thrombolysis and the extent of incorporated α_2-PI into the fibrin clot collected from the plasma of individuals with acute ischemic stroke (AIS). Despite our knowledge derived from experimental stroke models, surprisingly, the extent of α_2-PI incorporation into clots has not been yet investigated in acute stroke patient cohorts undergoing thrombolysis, where it may be associated with clinical outcomes.

Previous studies on the risk of venous thrombosis, where only α_2-PI activity levels were measured, did not provide convincing results on the role of α_2-PI. The determination of the C-terminally cleaved forms of α_2-PI and their proportions in thrombotic diseases have so far been addressed by only one study, in which levels of C-terminally intact and truncated forms in men with myocardial infarction (MI) were compared with an age-matched control group. Only a few studies have investigated the relationship between the α_2-PI p.Arg6Trp polymorphism and arterial thrombosis. The assumed protective effect of the p.Arg6Trp polymorphism based on biochemical studies could not be demonstrated in patients with either coronary atherosclerosis or AIS. The effect of the polymorphism in venous thrombosis has not been studied so far.

2. REVIEW OF LITERATURE

Thrombotic diseases are playing the leading role in both mortality and morbidity. Thrombosis can occur in both arterial and venous circulation. Arterial occlusion can mainly cause affects myocardial infarction (MI), or acute ischemic stroke (AIS), while obstruction of venous circulation leads to deep vein thrombosis (DVT).

2.1. Venous thromboembolism

Venous thromboembolism (VTE) is a disease classification that includes clots that have formed in the veins of the legs and arms, known as deep vein thrombosis (DVT), as well as clots that have embolized and traveled to the lungs, known as pulmonary embolism (PE). The incidence rate of VTE is estimated to be 1 to 2 per 1,000 person-years among persons of European ancestry, In 60% of VTE cases, only DVT occurs, while in 40% of cases PE occurs with or without DVT. VTE is a multifactorial cardiovascular disease, both environmental and genetic risk factors contribute to VTE risk. Environmental risk factors can be classified as provoking (e.g. cancer, surgery, trauma or fracture, immobilization, pregnancy and the postpartum period, long distance travel, hospitalization, catheterization, and acute infection) or non-provoking (e.g. age, sex, race/ethnicity, obesity, oral contraceptive, or hormone therapy use, corticosteroid use, statin use, diet, physical activity and sedentary time, and air pollution) factors. Other factors that can increase the risk of VTE are: transvenous pacemaker placement, DM, hypertension, previous family history of VTE and varicose veins, previous superficial vein thrombosis, neurologic disease with extremity paresis, elevated levels of triglycerides and low high-density lipoprotein (HDL).

The genetic basis of VTE is only partly understood. VTE inheritance follows a multifactorial, or non-Mendelian, inheritance model. At present 20–30 genetic VTE risk factors are known, and most of the well characterized genetic risk factors involve mutations of clotting factors, which include variants in factor V, prothrombin, fibrinogen gamma, and blood group non-O. These mutations increase VTE risk about 1.5 to 3-fold. In summary, both environmental and genetic factors contribute to the risk of VTE.

2.2. Acute ischemic stroke

Stroke is the leading cause of long-term disability in developed countries and one of the top causes of mortality worldwide. The incidence of the disease and the number of death are the highest in Eastern Europe and also in Hungary it is the third most common cause of death. Approximately 80% of strokes are caused by focal cerebral ischemia due to arterial occlusion, while up to 20% are caused by intracerebral hemorrhages. In patients with AIS, rapid restoration of blood flow is essential to reduce the rate of disability. The solution to the blockage of blood flow is to dissolve the thrombus, which today has only one pharmacological treatment: intravenous recombinant tissue plasminogen activator (rt-PA) received within 3-4.5 hours after the onset of symptoms (therapeutic time window). Although this therapy has been proven to be safe and effective in a number of clinical trials and meta-analyses, it is not a remedy for all. Unfortunately, due to the narrow time window, not all patients who have had a stroke can receive appropriate treatment. In the majority of patients (~50-60%), thrombolysis is less effective due to the failure of recanalization, and clinical improvement will not occur. Moreover, in approximately 6–8% of patients, therapy-associated bleeding complications will take place, which potentially leads to worsening of symptoms and might be fatal in approximately 1% of thrombolysed patients.

Risk factors for AIS may include gender, age, ethnicity, family history, genetic factors, hypertension, DM, atrial fibrillation, smoking, ischemic heart disease, hyperlipidemia, dyslipidemia, excessive alcohol consumption, hormone therapy or drug use, etc. World stroke organizations have jointly developed a scale system, the National Institutes of Health Stroke Scale (NIHSS) based on which we may be able to objectively determine the severity of the stroke. The scale system takes into account several aspects, which are as follows: aphasia, dysarthria, neglect, level of consciousness, answers to various questions (age, date), executing various instructions, field of view, eye movement, facialis paresis, muscle strength in the lower and upper limbs, muscle strength in the lower and upper limbs (limb ataxia) and awkwardness. The outcome and safety of thrombolysis is likely to depend on factors influencing or regulating fibrinolysis, but the exact pathomechanism is largely unknown.

2.3. Fibrinolysis

The fibrinolytic system plays an important role in the dissolution of blood clots and in the maintenance of a patent vascular system. Plasminogen an inactive proenzyme, that can be

converted to the active enzyme plasmin is a key component of this system. Plasmin degrades fibrin into soluble fibrin degradation products. Plasminogen is a serine protease produced by the liver.

Two types of plasminogen activators have been identified in the blood: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA).

Inhibition of the fibrinolytic system may occur at the level of plasminogen activation, mainly by a specific plasminogen activator inhibitor (PAI-1) or by thrombin-activatable fibrinolysis inhibitor. Inhibition of the fibrinolytic system can also be mediated through inhibition of plasminogen activators as well as plasmin. Inhibition of plasminogen activation is mediated by plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2).

FXIII is a pro-transglutaminase that circulates in plasma in tetrameric form (FXIII-A_2B_2). It consists of two potentially active, catalytic A subunits (FXIII-A) and two carrier/inhibitory B subunits (FXIII-B). FXIII becomes activated in the final phase of coagulation cascade by thrombin and Ca^{2+}. The active form (FXIIIa) is a transglutaminase that forms ε(γ-glutamyl)lysyl crosslinks between two polypeptide chains. An important function of hemostasis is the cross-linking of fibrin α- and γ-chains in which it forms γ-chain dimers and high molecular weight α-chain polymers. Another important function is the cross-linking of the fibrinolysis inhibitor α2-PI to the α-chain of fibrin, forming a covalent crosslink between Gln14 of α2-PI and Lys303 on the α-chain of fibrin.

2.4. α_2-plasmin inhibitor (α_2-PI)

α_2-PI is the main physiological inhibitor of plasmin. The human α_2-PI is a single-chain glycoprotein, which is a serine-protease inhibitor, the member of the serpin superfamily. Unlike other serpins, α_2-PI at the C-terminal end is approximately it contains 50-55 amino acids more. It is able to bind plasmin(ogen) through the lysines in this area. Its concentration in human plasma is ~1 μM (≈70 mg/L), and the in vivo half-life is ~2.6 days. It is primarily synthesized and secreted by the liver, but also can be produced in smaller amounts by the kidneys and the brain. Its molecular weight is ~67 kDa and its carbohydrate concentration is ~11-14% and contains sulfated tyrosine residues at position 457. The primary physiological function of α_2-PI is the inhibition of fibrinolysis by forming an irreversible inactive complex with plasmin. The complex formation is a two steps process, first, the C-terminus of α_2-PI

reversibly interacts with the Lys-binding sites of plasmin(ogen), then a covalent bond is formed between the reactive site of α_2-PI and the active site of plasmin.

The gene coding for human α_2-PI, SERPINF2, is located on chromosome 17p13.3 and encodes a 27 amino acid residue signal peptide and a single-chain protein of 464 amino acid residues. The gene contains 10 exons and 9 introns and spans approximately 16 kb of DNA. The sequence of SERPINF2 shows 23% to 28% homology with other members of the serpin family and 73% to 81% homology with α_2-PI from other species.

2.4.1. N-terminal variation of α_2-plasmin inhibitor

In the plasma the full-length α_2-PI is proteolytically modified, leading to a variety of circulating α_2-PI forms. About 70% of plasmatic α_2-PI is N-terminally cleaved between the proline residue at position 12 and the asparagine residue at position 13. This results in an α_2-PI molecule with an asparagine (Asn) residue at the N terminus (Asn- α_2-PI). The remaining 30% is non-truncated and circulates in plasma with a methionine (Met) residue at the N terminus (Met- α_2-PI). This N-terminal cleavage has been shown to affect the cross-linking of α_2-PI to fibrin by FXIIIa.

The arginine-to-tryptophan (Trp) polymorphism (p.Arg6Trp, rs2070863) is a functional polymorphism affecting the conversion of Met- α_2-PI to Asn- α_2-PI and thereby the rate of α_2-PI incorporation into fibrin. It has been shown that plasma purified Met- α_2-PI (Arg6) was cleaved approximately eightfold faster by antiplasmin-cleaving enzyme/sFAP than Met- α_2-PI (Trp6).

2.4.2. Soluble fibroblast activation protein (sFAP)

Soluble FAP is a member of the dipeptidyl peptidase (DPP) 4 family. Among the members of the DPP family, FAP has endopeptidase activity in addition to dipeptidyl peptidase activity. FAP is found in two forms in the human body, soluble and membrane-bound. FAP is a type II integral membrane protein. Its soluble form is found both intra- and extracellularly.

Soluble FAP is able to cleave the denatured form of type I collagen and α_2-PI through endopeptidase activity, therefore, it is also called an antiplasmin cleaving enzyme (APCE). This enzyme cleaves the first 12 amino acids in the N-terminal part of α_2-PI. FAP is produced by activated fibroblasts during wound healing and embryogenesis and has also been shown to be expressed in epithelial-derived cancers.

Previously, sFAP antigen levels have been studied in different patient groups. A commercially available sandwich ELISA method was used for this determination. sFAP antigen levels was measured in patients with acute coronary heart disease and healthy controls Significantly higher sFAP antigen concentrations were found in this group of patients compared to the control group, and higher values were measured in men with acute coronary heart disease than in women. In a follow-up study, 29 of 407 patients died in the first year of recovery, who had much lower sFAP levels than those who survived. Based on these results, it was concluded that low sFAP concentrations increase the risk of mortality in the group of patients with acute coronary heart disease. Another research groups, also looked at sFAP antigen levels in coronary heart patients, AIS patients as well as peripheral arterial disease. No significant difference in sFAP levels was found between patients with arterial thrombosis and controls in contrast, although lower sFAP concentrations were found in patients with coronary artery heart disease, but this was not significant compared to controls. Examining gender sFAP levels in the control group, it was found that men had higher plasma protein levels than women. sFAP levels showed a significant correlation with BMI, suggesting that elevated BMI is associated with high sFAP antigen concentration. In another study examined the association of sFAP levels with the severity of liver cirrhosis. Elevated sFAP concentrations were found in patients compared to controls and this increase was significantly correlated with the severity of liver cirrhosis. sFAP levels have not been studied in patients with venous thrombosis.

2.4.3. C-terminal variation of α_2-plasmin inhibitor

The C terminus of α_2-PI is post-translationally modified. The total α_2-PI is approx. 35% truncated on this end. The in vivo cleavage site was published in 2020 stating that the major cleavage site may be between glutamine 421 and aspartic acid 422, but other potential cleavage sites have also been identified. α_2-PI binds to the Kringle domains of plasminogen via lysines at the C-terminus, but in the absence of the C-terminus, it is no longer able to bind plasminogen and thus inhibits plasmin more slowly. The enzyme that cleaves has not yet been identified. C-terminally truncated α_2-PI is at least 26 amino acids shorter than the full-length α_2-PI.

Besides the lysine residues, the C-terminal region of human α_2-PI also contains an arginine-glycine-aspartic acid (RGD) sequence, that is crucial for cell recognition and

adhesion via integrins. The functionality of this RGD sequence in α_2-PI has only marginally been studied.

3. AIMS

Although α_2-PI plays an important role in the regulation of fibrinolysis and thus in thrombotic diseases by inhibiting plasmin, and based on previous studies we hypothesize that only α_2-PI bound to the clot by FXIIIa effectively inhibits fibrinolysis, little is known about the factors influencing the incorporation of α_2-PI into the clot and their clinical consequences. There is also little information available on the amount and role of different proteolytically modified α_2-PI forms in the plasma in various thrombotic diseases.

1. We developed a method for the determination of the amount of α_2-PI incorporated into the clot obtained *in vitro* from citrated plasma and investigated the effect of FXIII plasma concentration on the incorporation.
2. We measured plasma concentrations of α_2-PI and sFAP antigen, and determined the α_2-PI p.Arg6Trp polymorphism in a group of patients with acute ischemic stroke and healthy controls.
3. We examined the incorporation of α_2-PI into the plasma clot and its association with the outcome of thrombolysis therapy in patients with acute ischemic stroke
4. We measured α_2-PI activity, total α_2-PI, PB-α_2-PI and NPB-α_2-PI antigen and sFAP antigen levels, and determined the α_2-PI p.Arg6Trp polymorphism in healthy controls and patients with venous thrombosis.
5. We investigated the correlation of α_2-PI antigen and activity levels with each other and with other parameters.
6. We examined whether high total α2-PI, PB-α2-PI and NPB-α_2-PI and sFAP concentrations increase the risk of venous thrombosis.
7. Since the proteolytic effect of sFAP is reduced by the α_2-PI p.Arg6Trp polymorphism, we investigated whether an interaction effect of the two parameters can be detected in influencing the risk of thrombosis.

4. MATERIAL METHODS

4.1. The Effect of Thrombin Concentration and Time on the Incorporation of α_2-PI into Fibrin Clots

The extent of α_2-PI incorporation into fibrin clots was studied using an in-house sandwich ELISA assay that measures all forms of α_2-PI and is not influenced by the presence of plasmin–antiplasmin complexes (reference range of plasma α_2-PI: 48-85 mg/L) (Teraz-Orosz, A., et al., J Immunol Methods, 2019. 471:27-33.). Healthy plasma samples (n = 10) were clotted using 2 U/mL thrombin (CoaChrom, Maria Enzersdorf, Austria) and 20 mM $CaCl_2$. After incubation at 37 ◦C for 30 min, serum samples, derived from the extrusion of fluid after plasma clotting, were separated by centrifugation (16,100 g, 5 min). α_2-PI antigen levels were measured from the plasma samples before clotting and from the obtained serum samples. The extent of α_2-PI incorporation into fibrin clots was calculated using the following formula:
α_2-PI incorporation (%)=(plasma α_2-PI [mg/L]-serum α_2-PI [mg/L])/plasma α_2-PI (mg/L) × 100

The effect of thrombin concentration on the extent of α_2-PI incorporation and its time dependence was tested using various amounts of thrombin (0.5, 1, 2, and 5 U/mL) and various clotting times (10, 20, 30, 45, 60, and 180 min).

4.2. The Effect of FXIII Concentration on the Incorporation of α2-PI into Fibrin Clot

The effect of FXIII levels on α_2-PI incorporation into the fibrin clot was investigated using a FXIII-deficient plasma sample with normal α_2-PI level. FXIII-deficient plasma aliquots were supplemented with various amounts of purified plasma FXIII (FXIII-A_2B_2) (2, 5, 10, 15, 20, 25, 30, 35, and 40 mg/L). Purified FXIII was isolated from pooled plasma of healthy individuals.

- After FXIII supplementation, plasma samples were clotted using 2 U/mL thrombin and 20 mM $CaCl_2$ for 30 min.
- Total α_2-PI antigen levels in the plasma and serum samples were measured as described above.
- Clots were washed in compact reaction columns with 20 × 500 µL PBS, pH: 7.2 containing 3 mg/mL iodeacetamide (IAA)

- Dissolved in Laemmli buffer containing 5% mercaptoethanol and 8 M urea at room temperature for 20 h.
- Dissolved clot samples were analyzed by SDS-PAGE on 7.5% polyacrilamide gels.
- Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) and immunostained with polyclonal anti-α_2-PI antibody (GA2AP-AP, Affinity Biologicals, ON, Canada) that was labeled with horseradish-peroxidase (HRP)
- Visualized using enhanced chemiluminescence detection (ECL, Thermo Fisher Scientific, Waltham, MA, USA) in Azure c300.

4.3. Patients and Controls

4.3.1. Acute ischemic stroke study

Fifty-seven acute ischemic stroke patients, all within 4.5 h of their symptom onset before i.v. thrombolysis treatment using rt-PA (Alteplase, Boehringer Ingelheim, Germany) and 26 age-matched healthy controls were recruited in the study. AIS patients were enrolled between September 2016–June 2017 in a single Stroke Center (Department of Neurology, Faculty of Medicine, University of Debrecen, Hungary). All patients received i.v. thrombolysis according to current guidelines, inclusion and exclusion criteria were identical to standard eligibility criteria. Patients who also underwent mechanical thrombectomy were excluded from the study. The presence of AIS was diagnosed based on neurological symptoms and non-contrast CT (NCCT) scan and CT angiography (CTA). Patients were grouped according to their short-term outcome based on the change in their functional neurological status (National Institutes of Health Stroke Scale score, NIHSS) as assessed on admission and at 7 days after thrombolysis. A decrease in the NIHSS score by at least 4 points or to 0 by day 7 was defined as a favourable outcome, while an increase in NIHSS score by at least 4 points was defined as a poor outcome. The presence of hemorrhagic transformation (intracranial bleeding) was defined according to ECASS II criteria based on a control NCCT performed at 24 h post-event. Peripheral blood samples were drawn from AIS patients on admission, before the initiation of rt-PA infusion. Routine laboratory examinations (ions, glucose levels, renal and liver function tests) were performed from serum samples according to standard protocols.

4.3.2. Venous thromboembolism study

Two hundred and eighteen non-related consecutive VTE patients were enrolled in the study who admitted to the Thrombosis Center of the University of Debrecen during the year 2014. Fasting blood samples were collected at least 3 months after the acute event. Patients with malignant disease, antithrombin, protein C and protein S deficiency were not included in the study. Deep vein thrombosis (DVT) was confirmed by colour Doppler ultrasonography or venography, pulmonary embolism (PE) was diagnosed according to the guidelines of the European Society of Cardiology.

The same number of age and sex-matched healthy volunteers served as controls recruited from the same geographical area and were enrolled with the help of family practitioners. All chronic diseases except for moderate hypertension (blood pressure between 145/90 and 165/95 mmHg) and any acute illness in the previous 3 weeks were considered as exclusion criteria for healthy controls.

The studies fully complied with the Declaration of Helsinki. All enrolled individuals gave written informed consent. Ethical approval was obtained from the Regional Ethics Committee at the University of Debrecen, Hungary: DE RKEB/IKEB: 4672-2016; ETT TUKEB: 54005-3/2016/EKU

4.3.3. Preparation of plasma samples

Blood was taken from the antecubital vein into vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ) containing 1/10 volume of 0.109 M citrate between 8 am and 11 am from VTE patients and controls and on admission, before the initiation of rt-PA infusion from AIS patients. Plasma was separated by centrifugation at 1500g for 20 min, and aliquots were stored at − 70 ◦C until measurements. Non-anticoagulated blood was collected to obtain serum samples.

4.4. Determination of total α_2-PI incorporated into the fibrin clot

- The Na-citrate plasma of 57 AIS patients and their age-matched 26 controls was clotted with 2 U / mL thrombin and 20 mM $CaCl_2$ in a 37 C for 30 min.
- Serum and clot were separated by centrifugation (16 000 *g*, 5 min)
- Total α_2-PI antigen levels were determined from the original plasma and the resulting serum by sandwich ELISA.

- The amount of total α2-PI incorporated into the clot was calculated from plasma and serum levels as we described in 4.1. topic.

4.5. PB-α_2-PI measurements

PB-α_2-PI antigen levels were determined by a new ELISA technique developed by our laboratory.

The PB-α_2-PI antigen ELISA assay:

- The surface of a 96-well plate (EB, Thermo Fisher Scientific) was coated with polyclonal goat total anti-α_2-PI antibody (GA2AP-AP) (3 μg/mL, 100 μL/well, in 0.2 M $NaHCO_3$, pH 9.6) and incubated overnight at + 4 °C.
- The plate was blocked with 150 μL/well dilution buffer (1% BSA, 0.1% Tween20-TBS (50 mM Tris, 100 mM NaCl, pH 7.4)) for 1 hour at room temperature.
- After blocking, a 100 μL/well-diluted standard, plasma or serum sample was applied to the plate and incubated for 1 hour at room temperature.
- Then, 100 μL of 0.5 μg / mL mouse monoclonal anti-human PB-α_2-PI antibody was applied to the wells of the plate and incubated for 1 hour at room temperature.
- 100 μL biotinylated goat anti-mouse IgG (Southern Biotech) was added to each well of the plate at 5000-fold dilution and also incubated for 1 hour at room temperature.
- Then, 100 μl of streptavidin-HRPO conjugate (Southern Biotech) was added to the wells at 3000-fold dilution and incubated for 30 minutes at room temperature.
- A solution of 100 μL of 3,3 ', 5,5'-Tetramethylbenzidine (TMB, One Component HRP Microwell Substrate, Diarect Ag, Freiburg, Germany) was added to the wells of the plate and incubated in the dark for 10 minutes at room temperature.
- After 10 min, the reaction was stopped by adding 50 μL/well 2 M H_2SO.
- The absorbance was read at 450 nm in a Labsystems iEMS MF microplate reader (Labsystems Oy, Helsinki, Finland).
- Between all incubation steps, wells were washed three times with TBS containing 0.05% Tween 20 (300 μL/well)
- Standard human plasma (Siemens) was used to calibrate the assay. The PB-α_2-PI concentration of the standard human plasma by definition was 0.65 U/L, thus total-α_2-PI concentration determined by ELISA was multiplied by 0.65 to get PB-α_2-PI (mg/L)

concentration. Diluted series of standard plasma in the range of 10–500 μg/L was used to create calibration curves.

- Commercial Normal and Pathologic controls and pooled normal plasma were used for the measurement of precision (1:1000(v/v) dilution).
- The samples were measured in duplicate and the results were given as the average of the two measurements.

NPB-α_2-PI was calculated by subtraction of PB-α_2-PI from total-α_2- PI.

4.6. Other laboratory parameters

- Total α_2-PI was measured by in-house sandwich ELISA.
- Functional fibrinogen levels were measured by the Clauss assay (Siemens Healthineers, Erlangen, Germany).
- High-sensitivity C-reactive protein (hsCRP) level was measured by routine method (Roche Diagnostics, Mannheim, Germany).
- FXIII-A_2B_2 antigen was determined by sandwich ELISA developed in our laboratory (Katona, E., et al., Thromb Haemost, 2000. 83(2):268-73.)
- DNA was isolated from the buffy coat of citrated blood samples by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).
- α_2-PI p.Arg6Trp (rs2070863) polymorphism was identified by real-time PCR on LightCycler® 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany).
- Factor V Leiden and FII 20210 G > A mutations were determined.
- Plasma sFAP antigen levels were determined using human FAP DuoSet ELISA Development kit (R&D System, Abingdon, UK) following the manufacturer's protocol.

4.7. Statistical analysis

The distribution of the data was tested by the Kolmogorov-Smirnov test. Normally distributed data are presented as mean ± SD, nonnormally distributed data as median (interquartile range). Independent sample t-test was used to test differences in means, Mann-Whitney U test for differences in ranks and $\chi 2$ test for differences in proportions between the study groups. To investigate the correlation of α2-PI activity levels with total-α_2-PI, PB-α2-PI and NPB-α_2-PI antigen levels bivariate correlation analyses were performed (Pearson Correlation for total-α_2- PI and PB-α_2-PI; Spearman's rho for NPB-α_2-PI, as this parameter did not show normal distribution). Multiple linear regression analyses were performed in the

control group to determine parameters independently associated with different α_2-PI forms and sFAP levels. For this analysis non-normally distributed variables were natural log-transformed to achieve normal distribution. Parameters independently associated with different α_2-PI forms and sFAP levels were used as covariates when adjusted levels were compared using analysis of variance (ANOVA). Bonferroni correction was used for multiple comparisons. To study the effect of total-α_2-PI, PB-α_2-PI, NPB-α_2-PI and sFAP levels on the risk of VTE, total-α_2-PI, PB-α_2-PI, NPB-α_2-PI and sFAP levels were grouped into tertiles based on the 33rd and 67th percentile value of the respective parameter in the control group. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated. Adjusted ORs were obtained by using a logistic regression model that included the investigated parameter and other parameters independently associated with the risk of VTE. BMI, cholesterol and CRP were included as continuous variables after natural log-transformation. The level of significance was 95% ($p < 0.05$). Statistical analyses were performed using SPSS software (SPSS 26.0 for Macintosh, Chicago, IL).

5. RESULTS

5.1. The Effect of Thrombin Concentration and Time on the Incorporation of α_2-PI into Fibrin Clots

In order to determine the extent of α_2-PI incorporation into fibrin clots, we developed a simple method based on the quantification of α_2-PI by ELISA in plasma and in the serum after clotting the plasma by thrombin. By subtraction, the extent of α_2-PI incorporation in healthy control samples was found to be 44.0 ± 4.6% (n = 10). The effect of thrombin concentration on the extent of α_2-PI incorporation and its time dependence was tested using various amounts of thrombin and various times allowed for clot formation. The maximum extent of α_2-PI incorporation was found to be approximately 45%. This level of incorporation was reached at relatively low thrombin concentrations (0.5–2 U/mL), and higher thrombin concentrations had no additional effect. Incorporation of α_2-PI into fibrin clots occurred relatively quickly in the presence of 2 U/mL thrombin. The extent of incorporation was already around 40% after 10 min, and the maximum extent of incorporation was reached after 30 min time allowed for clot formation.

5.2. The Effect of FXIII Concentration on the Incorporation of α_2-PI into Fibrin Clots

The effect of FXIII levels on the incorporation of α_2-PI into fibrin clots was tested using FXIII-deficient plasma supplemented with various amounts of purified FXIII-A_2B_2. As opposed to early reports, where the effect of very low amounts of FXIII was studied on the extent of α_2-PI incorporation, here we show that increasing the amounts of FXIII above 8% (mg/L) indeed has additional effect on the extent of α_2-PI incorporation. Submaximal extent of incorporation (~40%) was reached in the presence of 21 mg/L (corresponding to 100%) FXIII-A_2B_2. Noticeably, increasing FXIII concentrations above this level had a minor additional effect on the extent of α_2-PI incorporation into fibrin clots. The effect of FXIII levels on the extent of α_2-PI incorporation into fibrin clots was also investigated by SDS PAGE and Western blotting for α_2-PI, after washing and dissolving the fibrin clots. Using this approach, it became evident that by increasing the concentration of FXIII in the plasma samples, the amount of cross-linked α_2-PI-fibrin α-chain polymers increase within the fibrin clots. This was detectable at concentrations of up to 30 mg/L FXIII. On the other hand, increasing FXIII concentration above this level resulted in highly crosslinked fibrin clots that

could not be dissolved using standard Laemmli buffer and therefore could not be investigated using this approach.

5.3. The Extent of α_2-PI Incorporation into Plasma Clots in Acute Ischemic Stroke Patients and Its Relation to Thrombolysis Outcome

In order to investigate whether the extent of α_2-PI incorporation into plasma clots is associated with thrombolysis outcome in acute stroke patients, the levels of fibrinogen, FXIII-A_2B_2 and the extent of and α_2-PI incorporation were measured from the plasma samples of 57 acute ischemic stroke patients, all within 4.5 h of their symptom onset before intravenous thrombolysis treatment and 26 age-matched healthy controls. Patients were grouped according to their short-term outcome. Patients experiencing therapy-associated intracranial hemorrhage were significantly older (72.2±10.0 vs 61.4±13.6; $p<0.01$) and had significantly more severe stroke based on admission NIHSS (10 (6.5-11.0) vs 1.0 (0.0-2.0); $p<0.01$) as compared to those with good outcome. FXIII levels were significantly lower in all patient groups as compared to healthy controls (23.4±5.5; 22.9±7.6; 21.3±6.7 vs 28.2±4.3 mg/L; $p<0.01$; good outcome, no change/poor outcome; therapy associated intracranial haemorrhage vs control). Fibrinogen levels were significantly increased in patients with therapy failure (no change/poor outcome) as compared to healthy controls (4.3±1.5 vs 3.6±0.5 g/L; $p<0.05$, respectively). It must be noted that admission CRP levels were significantly increased in patients with no change/poor outcomes and in patients with therapy-associated intracranial hemorrhage as compared to healthy controls (4.9 (2.8-7.1); 5.3 (2.5-11.9) vs 1.7 (0.8-3.4) mg/L; $p<0.01$, respectively). In patients with post-lysis intracranial hemorrhage, plasma α_2-PI levels were significantly lower as compared to healthy controls (52.8±19.7 vs 66.9±8.7 mg/L; $p<0.05$). The extent of α_2-PI incorporation into fibrin clots was significantly lower in the total cohort of patients as compared to healthy controls (41.6±11.3 vs 49.4±4.6 mg/L; $p<0.001$). When patients were grouped according to thrombolysis outcomes, the extent of α_2-PI incorporation was found to be significantly lower in patients with no change/poor outcomes and patients with post-lysis intracranial hemorrhage (41.5 ± 11.8% and 37.3 ± 14.0%) as compared to healthy controls (49.4 ± 4.6%). The extent of α_2-PI incorporation in patients with good outcome (47.4 ± 6.7%) did not differ significantly from that observed in controls. On the other hand, the extent of α_2-PI incorporation was significantly lower in those who suffered post-lysis intracerebral hemorrhage (37±14.5%) as compared to those with good outcomes (47.4±6.7%).

In controls, fibrinogen levels (r=0.453, p=0.039) showed a significant positive correlation with the extent of α_2-PI incorporation. In patients, a significant positive correlation was found between plasma α_2-PI levels (r=0.544, p<0.001), FXIII levels (r=0.303, p=0.022) and the extent of α_2-PI incorporation into fibrin clots. Moreover, a highly significant negative association was found between NIHSS on admission (r=-0.449, p=0.001) in patients and the extent of α_2-PI incorporation. This suggests that in case of more severe strokes, less α_2-PI is incorporated into fibrin clots in vitro. As the extent of α_2-PI incorporation has its limit (45–50%), the most likely reason for this association is that the fraction of α_2-PI that could be incorporated into fibrin clots is less available in the plasma samples of patients with more severe strokes due to considerable in vivo consumption. The negative association between NIHSS and the extent of α_2-PI incorporation was particularly strong in the subgroup of patients with post-lysis intracerebral hemorrhage ($r = -0.627$, $p = 0.039$).

sFAP levels were significantly lower in patients as compared to healthy controls (73.2±16.6 vs 90.5±21.2 μg/L; p<0.001). α_2-PI p.Arg6Trp polymorphism had no influence on outcomes and its allele frequency was essentially the same in controls and patients.

5.4. Effect of α_2-PI in venous thromboembolism patients

The median age of the sex and age-matched 218 patients and 218 controls were 40 years, and the number of men and women was close to equal (105 and 113). Among the 218 VTE individuals 44 patients suffered from both DVT and PE, in 9 cases only PE could be detected.

Risk factors for vascular diseases such as BMI (29.0 (25.9-32.9) vs 25.2 (21.8-28.7) p<0.001; patients vs control), hypertension (104 vs 45, p<0.001), hypercholesterolemia (156 vs 165; p<0.001), FV Leiden mutation (allel frequency: 19.7% vs 5.7%; p<0.001) were more prevalent in patients than in control subjects. The number of current smokers was lower in the patient group (32 vs 48; p=0.002). This is probably due to the fact that many patients quit smoking after the VTE event. Other acquired risk factors identified in the patient group were: oral contraceptive or hormone therapy use (19 cases), trauma (13 cases), long-distance travel (8 cases), surgery (7 cases), postpartum period (5 cases), lupus anticoagulant (3cases), corticosteroid use (2 cases), cancer (2 cases), pregnancy (2 cases), varicositas (2 cases). C-reactive protein (3.8 (2.3-6.7) vs 1.6 (0.9-3.5) mg/L; p<0.001; patients vs control), fibrinogen (3.7±0.66 vs 3.4±0.58 g/L; p<0.001) and plasma FXIII activity (119.6±27.4 vs 109.5±24.8%; p<0.001) and antigen (FXIII-A_2B_2) levels (117.8±27.4 vs 107,2±23.6%; p<0.001) were significantly higher in patients than in controls.

5.5. Correlation between α_2-PI activity and antigen levels

The highest positive correlation was found between α_2-PI activity and total α_2-PI antigen levels (r=0.690, p<0.001) in the control group. The PB-α_2-PI antigen also showed a strong correlation with the activity levels (r=0.562, p<0.001), however, between NPB-α_2-PI antigen and activity levels only a moderate correlation could be detected (r=0.394, p<0.001). In the patient group, a similar pattern was found: r=0.782 for total α_2-PI antigen; r=0.610 for PB-α_2-PI antigen and r=0.477 for NPB-α_2-PI antigen levels; (p<0.001). There was no significant correlation between α_2-PI activity and the percentage of NPB-α_2-PI (r=0.121, p = 0.107).

5.6. Correlations between α_2-PI activity and antigen levels and other parameters in the control group

α_2-PI activity, total α_2-PI antigen and PB-α_2-PI antigen levels moderately but significantly correlated with fibrinogen (r=0.289, p<0.001; r=0.295, p<0.001; r=0.242, p=0.002) and total cholesterol levels (r=0.238, p=0.002; r=0.233, p=0.003; r=0.253, p=0.001). A weak correlation was obtained with BMI, but this association was statistically significant only for total α_2-PI antigen (r=0.190, p=0.014). α_2-PI activity (r=-0.226, p=0.004), total α_2-PI (r=-0.157, p=0.043) and PB-α_2-PI (r=-0.199, p=0.010) showed a moderate negative association with age. The negative association of PB-α_2-PI with gender (r=-0.156, p=0,045) means that men tend to have lower levels as compared to women. To our surprise, NPB-α_2-PI did not correlate with the abovementioned parameters; it only showed a weak correlation with CRP levels (r=0.187, p=0.015) and hypertension (r=0.193, p=0.012).

5.7. α_2-PI activity and antigen levels in patient and control group

α_2-PI activity (130±15.6 vs 117±14.5%; p<0.001; patients vs control) and total α_2-PI antigen levels (72.7±9.2 vs 65.1±7.7 mg/L; p<0.001) were significantly elevated in VTE patients. Adjustment for fibrinogen, BMI, cholesterol, and age did not modify significantly the results. The mean value of C-terminally intact PB-α_2-PI did not differ significantly between patients and controls (41.8±5.6 vs 42.3±4.9 mg/L; p=0.295) however, the adjusted mean was slightly decreased in the patient group and the difference was statistically significant (41.2 (40.5-41.9) vs 42.6 (41.9-43.4) mg/L; p=0.013).

NPB-α_2-PI level was also significantly elevated in the patient group as compared to controls (30.8 (26.3-35.5) vs 21.9 (18.0-26.9) mg/L; p<0.001) and adjustment did not modify significantly the result. Women had higher PB-α_2-PI antigen (women vs men; 43.4±5.5 vs

40.0±5.2 mg/L; p<0.001; 43.3±4.6 vs 41.3±4.7 mg/L; p=0.02, in patients and control) and lower NPB-α_2-PI antigen levels in both groups (women vs men; 29.5 (25.8-35.3) vs 31.5 (26.9-35.6) mg/L; p=0.205; 21.2 (17.5-26.2) vs 23.6 (18.7-28.0) mg/L; p=0.061, in patients and control), but the difference was statistically significant only for PB-α_2-PI antigen level. The percentage of NPB-α_2-PI antigen level (women vs men; 41.1±5.7 vs 43.6±7.1%; p=0.003; 33.5±7.4 vs 35.8±6.3%; p=0.012, in patients and control) was significantly higher in men in both groups. The elevation of α_2-PI activity (women vs men; 134±14.1 vs 125±15.5%; p<0.001) and total α_2-PI (women vs men; 73.9±8.9 vs 71.5±9.1 mg/L; p<0.001) and PB-α_2-PI antigen levels (women vs men; 43.4±5.5 vs 40.0±5.2 mg/L; p<0.001) were more prominent in women VTE patients.

5.8. sFAP antigen levels

sFAP antigen levels showed a weak positive correlation with BMI (r=0.219; p=0,004) and FXIII activity levels (r=0,202; p=0,009) and a weak negative association with fibrinogen level (r=-0.160; p=0.039) in the control group. The median value of sFAP was significantly elevated in the VTE group (80.5 (67.0-99.8) vs 76.3 (65.3-90.1) µg/L; p=0.044), however, the difference was not statistically significant after adjustment for BMI, fibrinogen and FXIII activity (83.4 (80.3-86.6) vs 79.8 (76.6-82.9) µg/L; p=0.123). Men had higher median sFAP level in the patient group than women (women vs men; 77.4 (64.8-94.4) vs 83.6 (69.3-103.1) µg/L; p=0.019), while in the control group there was no statistically significant difference between sFAP median values of men and women (74.3 (63.3-87.6) vs 78.6 (69.1-92.4) µg/L; p=0.073).

5.9. Association of α_2-PI p.Arg6Trp polymorphism and sFAP antigen levels with the risk of VTE

The effect of α2-PI p.Arg6Trp polymorphism and sFAP antigen level on the risk of VTE was investigated. The genotype distribution of the polymorphism was consistent with the Hardy-Weinberg Equilibrium in the study population ($\chi 2 = 0.2284$, p = 0.6327). Trp6 carrier and allele frequencies did not differ significantly between patients and controls. In the whole study population, there was no significant association between possession of the Trp allele and VTE (OR: 0.912, 95% CI: 0.617–1.348), p = 0.645). Individuals with sFAP levels in the upper tertile (n = 90/72 in the patient/control groups) did not have a significantly higher risk for VTE (OR: 1.246, 95% CI: 0.793–1.959, p = 0.340), as compared to individuals with sFAP

levels in the lowest tertile (n = 71/ 70 in the patient/control groups), however carrying the Trp6 allele in patients with sFAP levels in the upper tertile (R6/W6 = 62/28 and 41/ 31 in the patient and control groups, respectively) exerted a significant protective effect against VTE, after adjustment for FV Leiden mutation, hypertension, BMI, CRP, fibrinogen and cholesterol (OR: 0.425, 95% CI: 0.199–0.911, p = 0.028).

5.10. Association of α_2-PI activity, total α_2-PI antigen, PB-α_2-PI antigen and NPB-α_2-PI antigen levels with the risk of VTE

α_2-PI activity (OR: 5.89, 95% CI: 2.714-12.806, p<0.001), total α_2-PI antigen (OR: 7.64, 95% CI: 3.459-16.896, p<0.001) and NPB-α_2-PI antigen levels (OR: 9.87, 95% CI: 4.095-23.783, p<0.001) in the upper tertile showed a strong association with the risk of VTE. After adjustment, OR values decreased in all cases but remained significantly high. PB-α_2-PI antigen levels in the upper tertile associated with a lower risk of VTE (OR: 0.77, 95% CI: 0.487-1.234, p=0.283), but the association became statistically significant only after adjustment for other confounding parameters (OR: 0.37, 95% CI: 0.173-0.762, p=0.007). Besides the effect of elevated antigen concentration, also an increase in the percentage of NPB-α_2-PI resulted in a significant increase in VTE risk (OR: 8.37, 95% CI: 3.635-19.259, p<0.001).

6.Discussion

It has been shown by elegant studies that the stability of the newly formed thrombi against premature fibrinolysis predominantly depends on the cross-linking of α_2-PI into the fibrin clot. Despite the great importance of this regulatory step and its potential influence on a wide range of thrombotic clinical events, surprisingly little is known on the extent of α_2-PI incorporation into the fibrin clot under various circumstances. We developed a new approach to test the extent of α_2-PI incorporation into fibrin clots by measuring total α_2-PI antigen levels from the plasma and the serum of plasma clots obtained after clotting. Using this method, the extent of α_2-PI incorporation in healthy control samples was found to be 44.0 ± 4.6%. In earlier studies, different approaches were used to detect α_2-PI in clots. Using SDS-PAGE and Western blotting of fibrin clots made from purified proteins to follow α_2-PI-fibrin α-chain crosslinking, approximately 30% of α_2-PI was reported to be incorporated into fibrin. A limitation of such studies is that purified α_2-PI used in the experiments was comprised of the plasminogen-binding form only. Moreover, Western blotting is not an optimal approach to quantify the amount of highly crosslinked α_2-PIfibrin α-chain polymers. In a more recent study, a similar approach to our method was used. The quantification of clot-incorporated α_2-PI was performed by ELISA in plasma and in the serum after thrombus formation, however, in that case thrombus formation was followed in a Chandler loop. By subtraction, the extent of incorporation into thrombi was found to be between 30–50%. Our method is less laborious as compared to thrombus formation in a Chandler loop, which allowed us to perform the investigations in a cohort of AIS patients and healthy controls. The extent of incorporation is not only the result of FXIIIa-mediated cross-linking of α_2-PI to fibrin, but to a somewhat lesser extent, the result of a non-covalent binding of α_2-PI to fibrin. The latter interaction has been implicated to potentially contribute to the proper orientation of α_2-PI and thus facilitate the cross-linking process. Our results provide evidence that as opposed to early findings, enhancing FXIII concentration above 8% results in elevated incorporation of α_2-PI into fibrin clots, and the maximal extent of α_2-PI incorporation is reached at FXIII levels above 100%. By studying the modifying effect of FXIII levels on the extent of α_2-PI incorporation in a wide-range of FXIII concentrations (0–200%), we were able to demonstrate that increasing FXIII levels up to supraphysiological levels result in gradual, extensive cross-linking of α_2-PI to the fibrin clot. This might represent a missing biochemical link related to clinical observations on the role of FXIII in acute thrombotic events. It has been published that elevated FXIII levels increase the risk of myocardial infarction in young adults and particularly in women. Reduced fibrinolytic capacity has been defined as a risk factor for

myocardial infarction and stroke in young patients, but the exact mechanisms have not been identified as yet. It has been presumed that elevated FXIII, by extensively cross-linking α_2-PI to the fibrin clot and effectively inhibiting fibrinolysis, could play a role in sustaining the occluding thrombus in circumstances when atherosclerosis is not as prominent. During thrombolytic treatment, regulatory steps inhibiting fibrinolysis must be overcome to achieve effective break-down of thrombi. It has been shown in animal models that the resistance of thrombi to t-PA-induced thrombolysis is greatly influenced by the amount of active α_2-PI contained in the newly formed thrombi. Inhibition of clot-bound α_2-PI enhanced thrombolysis significantly in a rabbit jugular vein thrombosis model and treating mice with an α_2-PI-inactivating antibody resulted in quick thrombus dissolution. Effects of elevated α_2-PI levels and its inhibition have been extensively studied in mice models of stroke thrombolysis and based on the results, proposals to enhance thrombolysis in acute stroke patients by inhibiting clot-bound α_2-PI have been suggested. Based on data derived from animal models, the potential association between thrombolysis outcome and the extent of α_2-PI incorporation into clots obtained from the plasma of acute ischemic stroke patients is intriguing. Despite our knowledge derived from experimental stroke models, surprisingly, the extent of α_2-PI incorporation into clots has not been yet investigated in acute stroke patient cohorts undergoing thrombolysis, where it may be associated with clinical outcomes. Our results show that in acute stroke patients within 4.5 h of their symptom onset, lower extent of *in vitro* α_2-PI incorporation was found in those with more severe stroke. Our results demonstrated a highly significant negative association between NIHSS on admission and the extent of α_2-PI incorporation. These results might be explained by a potential, considerable consumption of the fraction of α_2-PI that can be incorporated into the clot. As the extent of α_2-PI incorporation has its limit (45–50%), we can surmise that the fraction of α_2-PI that could be incorporated into fibrin clots is less available in the plasma samples of patients with more severe strokes due to significant *in vivo* consumption. It has been known for a long time that more severe strokes are associated with less favorable thrombolysis outcomes and lysis resistance, together with a higher chance of bleeding complications, but the exact reason for these associations has not been clarified. Here we show that the extent of *in vitro* α_2-PI incorporation into plasma clots was significantly lower in patients who suffered post-lysis intracranial hemorrhage as compared to those with favorable outcome. Similarly to what has been known from the literature, patients with therapy-associated intracranial hemorrhage presented with a significantly more severe stroke on admission (higher NIHSS) in our cohort. More severe strokes might be associated with larger thrombus burden, higher extent of α_2-PI incorporation

and thus lower susceptibility to thrombolysis together with an increased risk of bleeding. In these cases, a lower extent of α_2-PI incorporation *in vitro* could implicate that *in vivo* the balance of fibrinolysis might be shifted towards bleeding at the surrounding sites of the thrombus. Due to the lower availability of clot-bound α_2-PI, protection against t-PA-induced hemostatic challenge might be diminished in these cases. Post-lysis intracerebral hemorrhage is a feared side effect of rt-PA induced thrombolysis, and despite its adverse effects on clinical outcomes, little is known about the exact pathomechanism as yet. Experimental results derived from patients' samples on admission showing association with future intracerebral bleeding events are potentially helpful to understand the background of bleeding events and to implement strategies for the prevention of such events. in our study, parameters influencing α_2-PI heterogeneity (sFAP and α_2-PI p.Arg6Trp polymorphism) were tested but we could not show an association between these factors and clinical outcomes. In the future, well-designed prospective studies are warranted to investigate the role of such parameters influencing α_2-PI heterogeneity in relation to the risk of arterial thrombotic events and outcomes.

Former studies on the association of α_2-PI and the risk of venous thromboembolism in which α_2-PI activity levels were measured provided inconclusive results. Some studies failed to show an association, while others revealed that although α_2-PI was positively associated with clot lysis time, there was no association between α_2-PI and risk of VTE after adjusting for BMI. The proteolytic variation of the α_2-PI C-terminus was investigated only in a single study on male myocardial infarction survivors and age-matched controls. In our study both α_2-PI activity and antigen measurements were performed: α_2-PI activity was measured by a commercial chromogenic assay; total α_2-PI antigen and PB-α_2-PI antigen levels were measured by in-house ELISAs developed in our laboratory. NPB-α_2-PI antigen levels were calculated from the results of total and PB-α_2-PI antigen ELISAs. In our study, VTE patients had 13% higher mean α_2-PI activity and 11.8% higher mean total α_2-PI antigen and 41% higher mean C-terminally cleaved NPB-α_2-PI antigen level as compared to controls, while the PB-α_2-PI antigen levels did not differ significantly. The percentage of NPB-α_2-PI antigen (i.e. C-terminal cleavage) obtained in our control population (34.6 ± 7.0%) equals the 35% that has been published previously and significantly elevated (43.3 ± 6.5%) in the VTE group. Both in the control and the VTE groups good correlations were obtained between the measured activity and total antigen levels as well as between activity and C-terminally intact PB-α_2-PI antigen levels. The correlation of the activity results with the NPB-α_2-PI antigen

levels was moderate, which confirms the assumption that the α_2-PI activity assay is mainly measure the levels of PB-α_2-PI. We did not find a significant correlation between the percentage of NPB-α_2-PI antigen and α_2-PI activity levels. To investigate the association between risk factors for vascular diseases and α_2-PI levels, multiple regression analyses were performed. α_2- PI activity, total α_2-PI and PB-α_2-PI antigen levels showed an independent positive association with BMI and total cholesterol and negative association with age. This result is in line with the published results. In contrast, NPB-α_2-PI did not show association with these parameters but were weakly associated with hypertension and CRP. This result suggests that proteolytic enzymes associated with inflammation/endothelial activation may be responsible for C-terminal cleavage of α_2-PI. After adjustment for the independently associated parameters, the differences in the mean values of α_2-PI activity, total-α_2-PI and NPB-α_2-PI antigen levels of controls and VTE patients remained significant, and the slight decrease of PB-α_2-PI levels in VTE patients comparing to controls became statistically significant. In our study population, elevated α_2-PI activity, total α_2-PI and NPB-α_2-PI antigen levels and elevated percentage of C-terminal cleavage (upper tertile vs lowest tertile) were independently associated with VTE risk; in contrast, elevated PB-α_2-PI levels represented a lower risk for VTE. The C-terminal end of the molecule is important in the initial reaction of α_2-PI with plasmin. Activated FXIII predominantly cross-links C-terminally intact PB-α_2-PI to the fibrin clot. Cross-linked α_2-PI retains its full inhibitory activity and makes fibrin resistant to digestion by plasmin. It has also been reported that NPB-α_2-PI could still form a complex with plasmin, although this is a slow reaction. Thus, C-terminal cleavage may have significant clinical consequences as removal of the plasminogen binding sequences strongly influences α_2-PI activity. Based on our current knowledge we cannot provide a clear answer to the question of whether the elevation of NPB-α_2-PI level without decreasing of PB-α_2-PI has functional consequences or is merely a side effect of ongoing pathological processes. Further studies are needed to investigate the functional consequences of increased C-terminal truncation of α_2-PI.

N-terminal heterogeneity of α_2-PI is influenced by the level of sFAP and by the p.Arg6Trp functional polymorphism in the SERPINF2 gene. Based on the results of biochemical investigations it was supposed that elevated sFAP level by increasing the amount of the N-terminally truncated Asn1-α_2-PI that is more effectively cross-linked by activated FXIII could increase the risk of thrombotic disorders. In contrast, as Met1-α_2-PI(Arg6) is cleaved ~8-fold faster by sFAP than Met1-α_2-PI(Trp6), the possessing of the Trp6 variant by decreasing the

N-terminal truncation and by this way decreasing the inhibition of fibrinolysis may have a protective effect. Only a few studies investigated the relation of p.Arg6Trp polymorphism to thrombotic diseases. Our present study is the first in which the relation of the p.Arg6Trp polymorphism to venous thromboembolism was investigated. In good agreement with the results of previous studies, the allele frequencies of p.Arg6Trp were 0.795/0.205 in the total study population. The Trp6 carrier and allele frequencies did not differ significantly between patients and controls and there was no association between Trp6 carriers and VTE. Soluble FAP levels and α_2-PI N-terminal cleavage were determined in arterial thrombotic patients including coronary heart disease (CHD), ischemic stroke and peripheral arterial disease and control individuals. Significant correlation was obtained between sFAP level and α_2-PI N-terminal cleavage. Median sFAP levels were similar in the combined arterial thrombotic patients and control individuals. However, sFAP levels were reduced in CHD in the first month after the event. The good correlation between sFAP levels and the extent of α_2-PI N-terminal cleavage was also confirmed in patients with liver cirrhosis. In our study, sFAP antigen levels showed a weak positive correlation with BMI and FXIII activity level and a weak negative association with fibrinogen level in the control group. It was reported earlier that individuals with hyperlipidemia had significantly increased sFAP levels and men had significantly higher sFAP levels than women, however, we did not find an association with total cholesterol levels and also did not find significant gender differences in our control group, but in the VTE group, men had higher median sFAP levels than women. The median value of sFAP was significantly elevated in the VTE group, however, after adjustment, the difference was not statistically significant. Individuals with sFAP levels in the upper tertile did not have a significantly higher risk for VTE. Considering that the p.Arg6Trp polymorphism significantly affects the N-terminal cleavage of α_2-PI by sFAP, we examined whether we could detect interaction in the effect of the two parameters. Indeed, after adjustment for other disease-associated confounding parameters, we were able to show for the first time the protective effect of the Trp6 allele in individuals with higher sFAP levels. The fact that we were able to detect the protective effect of the polymorphism at high sFAP levels indirectly confirms that sFAP has an effect on VTE risk. However, the extent of crosslinking to fibrin and its effect on fibrinolysis is also influenced by many other factors, such as the levels of α_2-PI, fibrinogen and FXIII, their polymorphisms etc. As a result of this, the direct effect of high sFAP levels on VTE risk would most likely be detectable only after involving larger number of cases with propensity matching for other confounding parameters.

7. SUMMARY

α_2-plasmin inhibitor (α_2-PI) is the main physiological inhibitor of plasmin, but the efficient inhibition of fibrinolysis is primarily based on α_2-PI covalently bound to fibrin by XIII (FXIII). It is a heterogeneous protein in the plasma due to N- and C-terminal cleavages that have functional consequences. The plasminogen-binding form (PB-α_2-PI) is converted to a non-plasminogen-binding form (NPB-α_2-PI) by C-terminal truncation. Soluble fibroblast activation protein (sFAP) cleaves off a 12 amino acids long N-terminal fragment; the resulting shortened inhibitor is a better substrate for FXIII. The rate of N-terminal cleavage is decreased by p.Arg6Trp polymorphism of α_2-PI.

In this work we investigated the factors modifying α_2-PI incorporation into the fibrin clot and whether the extent of incorporation has clinical consequences. We also investigated the heterogeneity of α_2-PI in venous thromboembolism (VTE) and the effect of the heterogeneity on VTE risk.

Unlike the previously reported results, FXIII levels even at levels above the upper limit of normal increased α_2-PI incorporation into the fibrin clot.

We measured fibrinogen, FXIII, and sFAP levels, α_2-PI incorporation into the *in vitro* plasma clot, and α_2-PI p.Arg6Trp polymorphism from samples of 57 acute ischemic stroke patients (AIS) before thrombolysis and 26 healthy controls. There was no significant difference between α_2-PI incorporation of controls and patients with good outcomes (49.4 ± 4.6% vs. 47.4 ± 6.7%, p = 1.000), however, in patients suffering post-lysis intracranial haemorrhage the incorporation was significantly lower (37.3 ±14.0%, p = 0.004). It seems that in the case of intravenous thrombolysis treatment of stroke patients α_2-PI incorporation shows an association with the outcome of therapy, particularly with intracranial haemorrhage. We also investigated total, PB- and NPB-α_2-PI antigen levels, α_2-PI activity, sFAP antigen levels and p.Arg6Trp polymorphism in 218 patients with VTE patients and 218 healthy controls. Total α_2-PI and NPB-α_2-PI levels were significantly elevated in VTE as compared to controls, while PB-α_2-PI levels did not differ. High total- and NPB-α_2-PI levels independently increased the risk of VTE (adjusted OR: 7.645; CI: 3.459–16.896 and 9.868; CI: 4.095–23.783, respectively). sFAP levels were significantly elevated in the VTE group. Neither elevated sFAP levels nor the α_2-PI p.Arg6Trp polymorphism alone affected the risk of VTE, however, we could demonstrate an interaction between the polymorphism and high sFAP levels, as in individuals with elevated sFAP levels the carriage of Trp6 allele associated with lower VTE risk.

MAIN SCIENTIFIC RESULTS, OBSERVATIONS

1. We have developed a method to determine the extent of incorporation of α_2-PI into the fibrin clot.
2. Here we provide evidence that as opposed to early findings, enhancing FXIII concentration above 8% results in elevated incorporation of α_2-PI into fibrin clots, and the maximal extent of α_2-PI incorporation is reached at FXIII levels above 100%.
3. Here we show that in acute stroke patients within 4.5 h of their symptom onset, lower extent of *in vitro* α_2-PI incorporation was found in those with more severe stroke.
4. α_2-PI activity, total α_2-PI and PB-α_2-PI antigen levels showed an independent positive association with BMI and total cholesterol level and negative association with age in the the healthy control group.
5. NPB-α_2-PI levels did not show association with these parameters but were weakly associated with hypertension and CRP levels.
6. After adjustment for the independently associated parameters, the differences in the mean values of α_2-PI activity, total α_2-PI and NPB-α_2-PI antigen levels of controls and VTE patients remained significant, and the slight decrease of PB-α_2-PI levels in VTE patients comparing to controls became statistically significant.
7. In our study population, elevated α_2-PI activity, total α_2-PI and NPB-α_2-PI antigen levels and elevated percentage of C-terminal cleavage (upper tertile vs lowest tertile) were independently associated with VTE risk; in contrast, elevated PB-α_2-PI levels represented a lower risk for VTE.
8. The Trp6 carrier and allele frequencies did not differ significantly between patients and controls and there was no association between Trp6 carriers and VTE.
9. The median value of sFAP was significantly elevated in the VTE group, however, after adjustment, the difference was not statistically significant. Individuals with sFAP levels in the upper tertile did not have a significantly higher risk for VTE.
10. We were able to show for the first time the protective effect of the Trp6 allele in individuals with higher sFAP levels.

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Registry number: DEENK/406/2021.PL
Subject: PhD Publication List

Candidate: Barbara Baráth
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Baráth, B.**, Kissné Bogáti, R., Miklós, T., Kállai, J., Mezei, Z. A., Bereczky, Z., Muszbek, L., Katona, É.: Effect of [alfa]2-plasmin inhibitor heterogeneity on the risk of venous thromboembolism.
   Thromb. Res. 203, 110-116, 2021.
   DOI: http://dx.doi.org/10.1016/j.thromres.2021.05.003
   IF: 3.944 (2020)
2. Bagoly, Z., **Baráth, B.**, Orbán-Kálmándi, R. A., Szegedi, I., Kissné Bogáti, R., Sarkady, F., Csiba, L., Katona, É.: Incorporation of [alfa]2-Plasmin Inhibitor into Fibrin Clots and Its Association with the Clinical Outcome of Acute Ischemic Stroke Patients.
   Biomolecules. 11 (3), 1-13, 2021.
   DOI: http://dx.doi.org/10.3390/biom11030347
   IF: 4.879 (2020)

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List of other publications

3. Zabczyk, M., Natorska, J., Bagoly, Z., Sarkady, F., **Baráth, B.**, Katona, É., Bryk, A. H., Zettl, K., Wisniewski, J. R., Undas, A.: Plasma fibrin clots of pulmonary embolism patients present increased amounts of factor XIII and alpha2-antiplasmin at 3 months' anticoagulation since the acute phase.
J. Physiol. Pharmacol. 71 (4), 519-524, 2020.
DOI: http://dx.doi.org/10.26402/jpp.2020.4.07
IF: 3.011

4. Bryk, A. H., Siudut, J., Broniatowska, E., Bagoly, Z., **Baráth, B.**, Katona, É., Undas, A.: Sex-specific alteration to α2-antiplasmin incorporation in patients with type 2 diabetes.
Thromb. Res. 185, 88-62, 2020.
DOI: https://doi.org/10.1016/j.thromres.2019.09.032
IF: 3.944

Total IF of journals (all publications): 15,778
Total IF of journals (publications related to the dissertation): 8,823

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

12 August, 2021

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