GRANT CLOSING REPORT

1. Background and aims

α2-plasmin inhibitor (α2-PI, α2-antiplasmin, SERPINF2) is a serine protease inhibitor; its primary physiological function is the rapid inhibition of plasmin by forming an irreversible plasmin-antiplasmin (PAP) complex. During coagulation, activated factor XIII (FXIIIa) cross-links α2-PI to the α-chain of fibrin. Cross-linked α2-PI retains its full inhibitory activity and makes fibrin resistant to digestion by plasmin. It has been reported earlier that only α2-PI cross-linked to fibrin can efficiently inhibit fibrinolysis. During circulation in plasma, α2-PI is modified by proteolysis leading to four different plasmatic forms. The N-terminal 12 amino acids of about 70% of α2-PI are removed by soluble fibroblast activation protein (sFAP, also named as APCE). This modification results in a variant, which is better substrate for FXIIIa than the full-length protein. This cleavage is modulated by the p.Arg6Trp polymorphism in the SERPINF2 gene (rs2070873), which results in a less efficient cleavage by sFAP. There are also C-terminally truncated variants of the α2-PI molecule, but the enzyme responsible for the cleavage is unknown. Lacking the C-terminal end α2-PI also lacks binding to plasminogen that strongly modulates its activity. It is assumed that the ratio of these differently modified molecular forms can strongly influences the fibrinolysis inhibitory effect of α2-PI. Our aim was to investigate the effect of molecular heterogeneity of α2-PI plasmatic forms on the risk of thrombotic disorders, to determine the amount of α2-PI incorporated into the clot by using different methods and try to identify the enzyme responsible for the C-terminal cleavage.

2. Major published results

2.1. The role of α2-PI activity, total α2-PI antigen level, C-terminally truncated α2-PI levels, soluble fibroblast activation protein (sFAP) activity and p.Arg6Trp polymorphism on the risk of venous thromboembolism (VTE)

We analysed the results of 218 non-related consecutive VTE patients admitted to the Thrombosis Centre of the University of Debrecen during the year of 2014. Same number of age and sex matched apparently healthy controls were also enrolled in the study. Fasting blood samples were collected at least 3 months after the acute event. Citrated plasma and serum sample aliquots were stored at -70°C until measurements. All chronic diseases except for moderate hypertension and any acute illness in the previous 3 weeks were considered as exclusion criteria for healthy controls. 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 European Society of Cardiology.

FXIII activity, α2-PI activity, fibrinogen, high-sensitive C-reactive protein (hsCRP) and total cholesterol levels were measured by routine laboratory methods. Plasma sFAP antigen levels were determined using human FAP DuoSet ELISA Development kit (R&D System, Abingdon, UK). α2-PI p.Arg6Trp (rs2070863) polymorphism was identified by real-time PCR.

FXIII-A_2B_2 antigen and total-α2-PI antigen were determined by sandwich ELISA methods developed by us previously. For the measurement of C-terminally non-truncated

(plasminogen binding, PB-α2-PI) α2-PI concentration a new ELISA was developed. C-terminally cleaved, non-plasminogen binding α2-PI (NPB-α2-PI) was calculated by subtraction of PB-α2-PI from total-α2-PI.
The following results were obtained:

α2-PI activity and total-α2-PI antigen levels were significantly elevated in VTE patients. Adjustment for fibrinogen, BMI, cholesterol and age did not modify significantly the results. The mean value of PB-α2-PI did not differ significantly between patients and controls, however, adjusted mean was slightly decreased in the patient group and the difference between patients and controls was statistically significant. NPB-α2-PI level was also significantly elevated in the patient group as compared to controls and adjustment did not modify significantly the result (Figure 1).

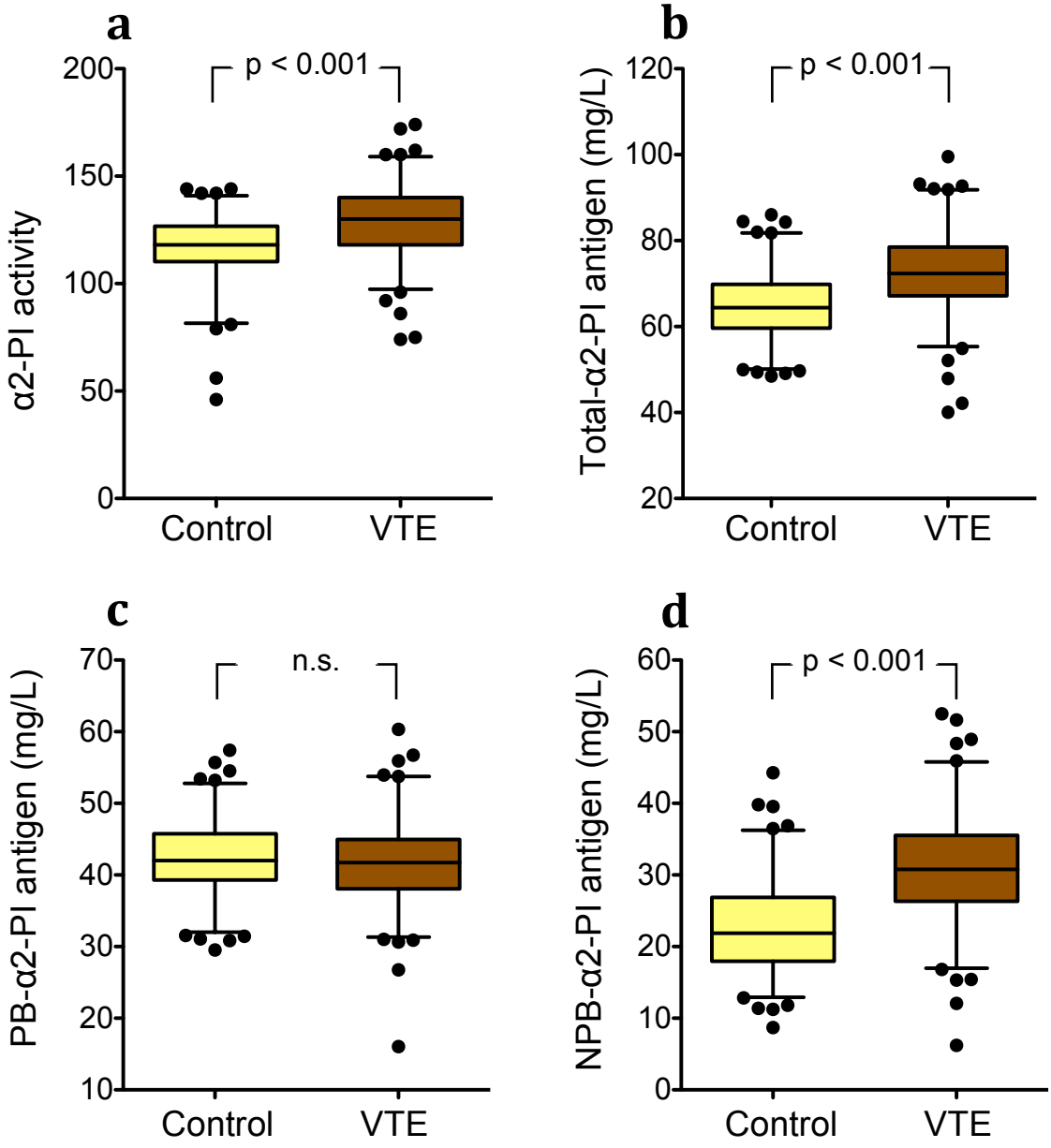

Figure 1
Non-adjusted levels of different α2-PI forms in control and VTE patient samples: (a) α2-PI activity; (b) total-α2-PI antigen; (c) PB-α2-PI antigen; (d) NPB-α2-PI antigen. The results are presented as boxes (median and the intervals between 25th and 75th percentiles) and whiskers (2.5th and 97.5th percentiles). n.s.: not significant

α2-PI activity, total-α2-PI antigen and NPB-α2-PI antigen levels in the upper tertile showed strong association with the risk of VTE, after adjustment for the parameters showing independent association with VTE (fibrinogen, hypertension, FV Leiden mutation, FII 20210 G > A mutation, cholesterol, BMI and CRP) the adjusted ORs (95% CI) were 5.895 (2.714-12.806), 7.645 (3.459-16.896) and 9.868 (4.095-23.783), respectively.

Median value of sFAP was significantly elevated in the VTE group (80.5 (67.0-99.8) vs. 76.3 (65.3-90.1)), however the difference was not statistically significant after adjustment for BMI, fibrinogen and FXIII activity. Individuals with sFAP levels in the upper tertile did not have significantly higher risk for VTE (OR: 1.246, 95% CI (0.793-1.959)), as compared to individuals with sFAP levels in the lowest tertile.

The genotype distribution of the α2-PI p.Arg6Trp polymorphism was consistent with the Hardy-Weinberg Equilibrium in the study population (χ^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).

In our study elevated sFAP level or carrying the α2-PI p. Arg6Trp polymorphism alone did not influence VTE risk, however, we were able to show an interaction between the two parameters; 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 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).

Our study was the first to investigate the molecular heterogeneity of α2-PI in VTE and also to demonstrate in a clinical study the hypothesized protective effect of the p.Arg6Trp polymorphism that was based on biochemical studies. Based on our current knowledge we cannot provide a clear answer to the question of whether the elevation of NPB-α2-PI level without decreasing of PB-α2-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 α2-PI. In our control group, NPB-α2-PI levels weakly associated with hypertension and CRP levels. The week association is probably due to the lack or low level of inflammation and low incidence of hypertension in the healthy control group. However, this result suggests that proteolytic enzymes associated with inflammation/endothelial activation may be responsible for C-terminal cleavage of α2-PI.

This work has been published: Baráth B, Bogáti R, Miklós T, Kállai J, Mezei ZA, Bereczky Z, Muszbek L, Katona É. Effect of α2-plasmin inhibitor heterogeneity on the risk of venous thromboembolism. Thromb Res 2021; 203:110-116.).

2.2. The effect of FXIII plasma level on α2-PI cross-linking to fibrin clot

In an early paper, it has been shown *in vitro* using purified conditions, that cross-linking of α2-PI by FXIIIa to the fibrin clot stops at about 30% incorporation, whereas in the same experiments the cross-linking of fibronectin reached almost 100%. It has been also published that enhancing FXIIIa concentration above 8% does not change maximal incorporation of α2-PI. However, FXIII concentration in the latter study was tested in sub-physiological levels only, and no follow-up papers were published using higher, physiological FXIII concentrations in the experiments.
We aimed to test the extent of α2-PI incorporation into fibrin clots by developing a new

approach, in which total α2-PI antigen levels from the plasma and from the serum of plasma clots are measured and compared. We studied the effect of thrombin concentration, the clotting time and the modifying effect of FXIII levels on the extent of α2-PI incorporation in a wide-range of FXIII concentrations (0-200%).

The effect of thrombin concentration on the extent of α2-PI incorporation and its time-dependence was tested using various amounts of thrombin and various times allowed for clot formation. The maximum extent of α2-PI incorporation was found to be approximately 45%. This level of incorporation was reached at relatively low thrombin concentrations (0.5-2 U/mL); higher thrombin concentrations had no additional effect. Incorporation of α2-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.

To study the effect of FXIII levels on the incorporation of α2-PI into fibrin clots FXIII deficient plasma was supplemented with various amounts of purified plasma FXIII (FXIII-A_2B_2) (Figure 2A and 2B). As opposed to early reports, we showed that increasing the amounts of FXIII above 8% (1,68 mg/L) indeed has additional effect on the extent of α2-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 α2-PI incorporation into fibrin clots. The effect of FXIII levels on the extent of α2-PI incorporation into fibrin clots was also investigated by SDS PAGE and Western blotting for α2-PI, after washing and dissolving the fibrin clots (Figure 2B). Using this approach, it became evident that by increasing the concentration of FXIII in the plasma samples, the amount of cross-linked α2-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 cross-linked fibrin clots that could not be dissolved using standard Laemmli buffer and therefore could not be investigated using this approach.

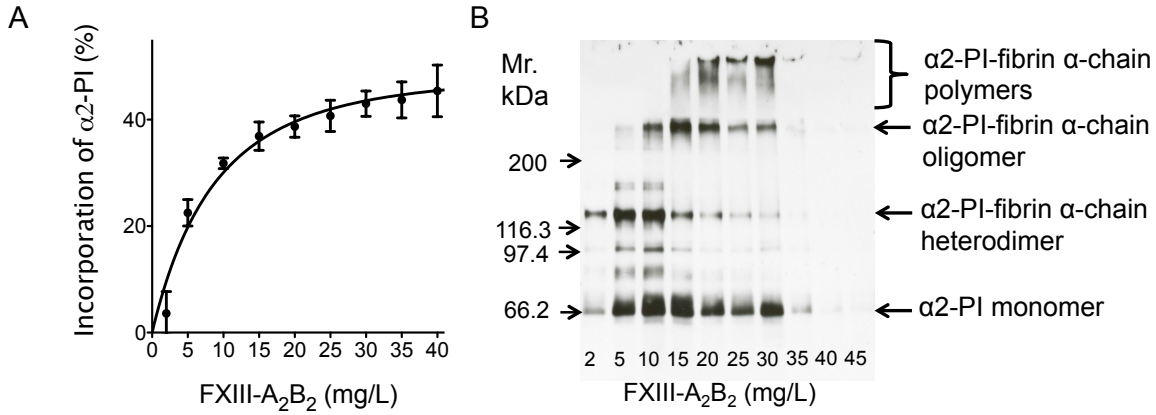

Figure 2. Effect of plasma FXIII level on the amount of α2-PI incorporated into the fibrin clot. FXIII deficient plasma sample (α2-PI = 62 mg/L) was supplemented with different amount of purified FXIII and clotted by thrombin and Ca^{2+}. (A) Differences of α2-PI antigen values between the substituted plasma and the respective serum were calculated and incorporation was presented as percentage of the respective plasma value.; (B) Fibrin clots were washed, dissolved and analyzed by SDS PAGE and Western blot using HRP-labeled polyclonal anti-α2-PI antibody and ECL detection.

Our results also showed that the extent of incorporation is not only the result of FXIIIa-

mediated cross-linking of α2-PI to fibrin, but to a somewhat lesser extent, the result of a non-covalent binding of α2-PI to fibrin as non cross-linked monomer α2-PI can also be seen on the Western blot. The latter interaction has been implicated to potentially contribute to the proper orientation of α2-PI and thus facilitate the cross-linking process. We provided evidence, that as opposed to early findings, enhancing FXIII concentration above 8% results in elevated incorporation of α2PI into fibrin clots, and the maximal extent of α2-PI incorporation is reached at FXIII levels above 100%. By studying the modifying effect of FXIII levels on the extent of α2-PI incorporation in a wide-range of FXIII concentrations (0-200%), we were able to demonstrate that increasing FXIII levels up to supra-physiological levels result in gradual, extensive cross-linking of α2-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 youngs, but the exact mechanisms have not been identified as yet. It has been presumed that elevated FXIII, by extensively cross-linking α2-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

This work has been published in: Bagoly Z, Baráth B, Orbán-Kálmándi R, Szegedi I, Bogáti R, Sarkady F, Csiba L, Katona É. Incorporation of α2-plasmin inhibitor into fibrin clots and its association with the clinical outcome of acute ischaemic stroke patients. Biomolecules **2021**, 11, 347. https://doi.org/10.3390/biom11030347

2.3. The role of α2-PI cross-linking to fibrin on the therapeutic outcome of acute stroke patients treated with thrombolysis

The extent of α2-PI incorporation into clots have not been investigated in patient cohorts of thromboembolic conditions, in which it may be associated with clinical outcomes. In our study ex vivo cross-linking of α2-PI to fibrin was investigated on 64 selected plasma samples of stroke patients undergoing intravenous thrombolysis treatment and 26 age-matched controls. Patients were grouped according to the clinical outcomes into three groups: favourable outcome (n=26), unfavourable outcome (n=20) and therapy-associated symptomatic or asymptomatic intracranial bleeding (n=12). Plasma samples obtained before thrombolysis were clotted by adding thrombin and Ca^{2+}. Total α2-PI antigen levels were measured from the serum and the original plasma samples by ELISA. The amount of α2-PI that incorporated into the clot was calculated by subtracting the value measured in the serum from the corresponding plasma value. Plasma FXIII A_2B_2 antigen and sFAP levels as well as A2-PI p.Arg6Trp polymorphism and the 50% clot lysis time were also determined.

The extent of α2-PI incorporation into fibrin clots was significantly lower in the total cohort of patients as compared to healthy controls (Figure 3A). When patients were grouped according to thrombolysis outcomes, the extent of α2-PI incorporation was found to be significantly lower in patients with no change/poor outcomes and patients with post-lysis intracranial haemorrhage (41.5±11.8% and 37.3±14.0%, respectively) as compared to healthy controls (49.4±4.6%). (Figure 3B). The extent of α2-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 α2-PI incorporation was significantly lower in those who suffered post-lysis intra-cerebral haemorrhage as compared to those with good

outcomes (Figure 3B). Median time to reach 50% clot lysis did not differ significantly between controls and patients (Figure 3C), moreover, 50%CLT did not show significant differences between controls and patients with different outcomes. Soluble FAP levels were significantly lower in patients as compared to healthy controls. α2-PI p.Arg6Trp polymorphism had no influence on the extent of α2-PI incorporation to fibrin clots in either groups.

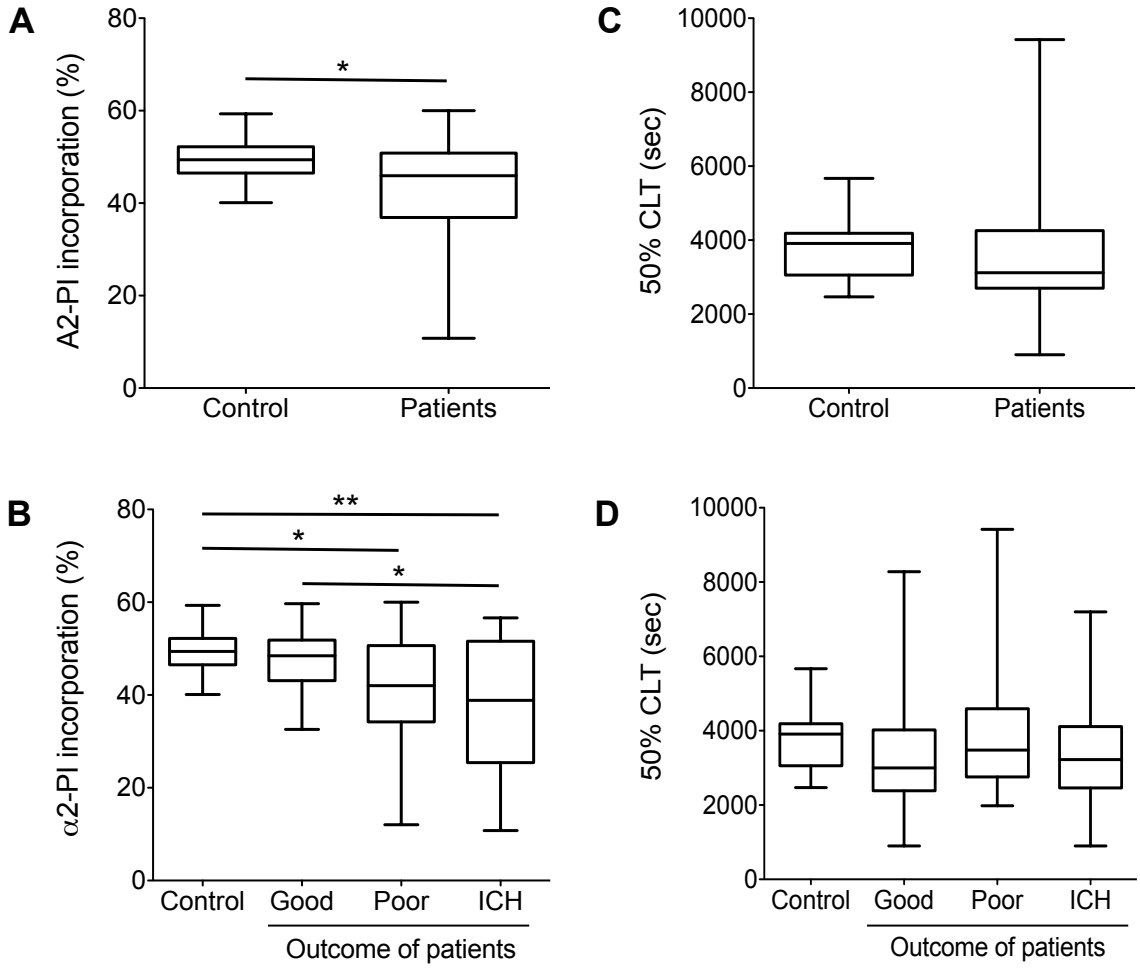

Figure 3. (A) α2-PI incorporation into the fibrin clot. Plasma samples were clotted by thrombin and Ca2+. Differences between plasma and serum α2-PI antigen values were calculated and incorporation was presented as percentage of the respective plasma values in controls and in the total cohort of patients (B) α2-PI incorporation into the fibrin clot in controls and in patients according to different outcomes (C) In vitro clot lysis experiments. The 50% CLT parameter is shown in controls and in the total cohort of patients. 50% clot lysis times (CLT) did not show significant differences between controls and patients (D) In vitro clot lysis experiments in controls and in patients according to different outcomes. 50% CLT did not show significant differences among patient groups. Box and whisker plots indicate median, interquartile range, and total range *, $p<0.05$; **, $p<0.01$

This work has been published in: Bagoly Z, Baráth B, Orbán-Kálmándi R, Szegedi I, Bogáti R, Sarkady F, Csiba L, Katona É. Incorporation of α2-plasmin inhibitor into fibrin clots and its association with the clinical outcome of acute ischaemic stroke patients. Biomolecules **2021**, 11, 347. https://doi.org/10.3390/biom11030347

The above-mentioned two publications were included in the PhD thesis of Barbara Baráth.

2.4. *α2-PI incorporation into fibrin clots of pulmonary embolism patients*

Reduced plasma FXIII levels have been reported in acute pulmonary embolism (PE) patients. In a collaboration study we investigated the impact of anticoagulant therapy on clot-bound amounts of FXIII and α2-PI and their associations with fibrin clot properties in patients with PE.

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

This work has been published: Zabczyk M, Natorska J, Bagoly Z, Sarkady F, Barath B, Katona E, Bryk A, 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., JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 71: (4) p. 1., 2020

2.5. *α2-PI incorporation into fibrin clots of patients with type 2 diabetes mellitus*

Type 2 diabetes mellitus (T2DM) is associated with hypofibrinolysis and it was recently published that factor XIII-mediated α2-PI incorporation into the fibrin clot is increased. However, it was unclear whether there are sex-related differences in α2-PI incorporation in relation to impaired clot lysis in T2DM.

In a collaboration study we investigated FXIII activity and α2-PI incorporation into fibrin clots as a determinant of clot lysability in a group of T2DM patients (48% women) using our ELISA methods.

Female patients had 15.2% greater α2-PI incorporation into the fibrin clot ($p=0.008$) and slightly higher plasma total α2-PI concentration ($p=0.005$) along with 8.4% longer time to 50% lysis ($p=0.012$) compared with men. Female patients had enhanced thrombin generation and higher endogenous thrombin potential ($p=0.003$) compared with men, while factor XIII activity was comparable between sexes ($p=0.085$). On multivariate regression, patient sex and glycated hemoglobin (HbA1c) level were the predictors of α2-PI incorporation in the entire patient group.

Our findings increase the current knowledge on factors affecting efficiency of fibrinolysis in patients with T2DM, showing an association between compromised fibrinolysis and increased α2-PI incorporation into the fibrin clot in female patients when compared with male subjects. Further studies are required to elucidate the mechanisms underlying this association.

This work has been published: Bryk A, Siudut J, Broniatowska E, Bagoly Z, Baráth B, Katona E, Undas A: Sex-specific alteration to alpha2-antiplasmin incorporation in patients with type 2 diabetes. Thrombosis Research 185: 55-62, 2020.

2.6. Measurement of total *α2-PI in different body fluids*
The ELISA method developed before the starting of this proposal was fully evaluated and published in this period.
Teraz-Orosz A, Csapo A, Bagoly Z, Szekely EG, Toth E, Kovacs B, Muszbek L, Katona É. A new ELISA method for the measurement of total alpha2-plasmin inhibitor level in human body fluids. J Immunol Methods. 2019 Aug;471:27-33.

3. Major results not yet published
The following works are closely related to the original proposal, the manuscripts are being prepared and will likely be published within a year.

3.1. Investigation of the incorporation of total-α2-PI and C-terminally truncated α2-PI (PB-α2-PI and NPB-α2-PI) into plasma clots

A) In the VTE study, we found that the amount of C-terminally truncated PI was higher in the VTE group as compared to the control group, and the elevated levels represented significant risk of VTE. Therefore, we aimed to investigate if the incorporation of the total- and different C-terminal forms of α2-PI into plasma clots of VTE patients and controls modified by the enhanced cleavage of α2-PI. Randomly selected citrated plasma samples from the control (n=86) and VTE (n=84) groups were clotted by thrombin and Ca^{2+} as described earlier. We measured total and PB- α2-PI antigen from the serum and the original plasma samples and the amount of different α2-PI forms that incorporated into the clot was calculated. Fibrinogen and FXIII levels were measured in the plasma earlier. Fibrin clots were also investigated by Western blotting. An in vitro clot lysis assay was performed using the platelet poor plasma samples. Clotting and subsequent lysis were induced by adding human tissue factor, rt-PA and $CaCl_2$. Optical density was measured at 340 nm 37 °C for 300 min. Curves were analyzed using the Shiny app software.
Main results:

- In the VTE samples there was no significant difference in fibrinogen levels, however, plasma FXIII, total-α2-PI and NPB-α2-PI antigen concentrations were significantly elevated, while PB-α2-PI levels were decreased as compared to controls.
- Interestingly, although total-α2-PI levels were significantly higher in the VTE group, the amount of total incorporated α2-PI was significantly lower in VTE samples compared to controls. The incorporation of PB-α2-PI was also significantly decreased, while the incorporation of NPB-α2-PI did not changed significantly (Fig. 4.)
- The significant reduction of PB- α2-PI incorporation in the VTE group is true when the amount of incorporated α2-PI is expressed as antigen concentration, the percentage of total incorporated α2-PI or the percentage of its original plasma concentration (Figure 5.).
- Incorporation of PB-α2-PI showed strong correlation with the level of plasma FXIII ($r=0.745$, $p<0.001$), while NPB-α2-PI incorporation did not show significant correlation in the control group ($r=0.053$, $p=0.629$).
- Western blot results showed non-covalent interaction of α2-PI with fibrin besides cross-linked products. Only cross-linked PB-α2-PI can be seen except two cases (2 and 9), where the plasma FXIII concentration was decreased (below 18 mg/L).

- Plasma total-α2-PI and NPB-α2-PI levels as well as the amount of incorporated NPB-α2-PI significantly correlated with 50% clot lysis time (CLT50) (r=0.313, p=0.003; r=0.387, p<0.001 and r=0.256, p=0.017, respectively).

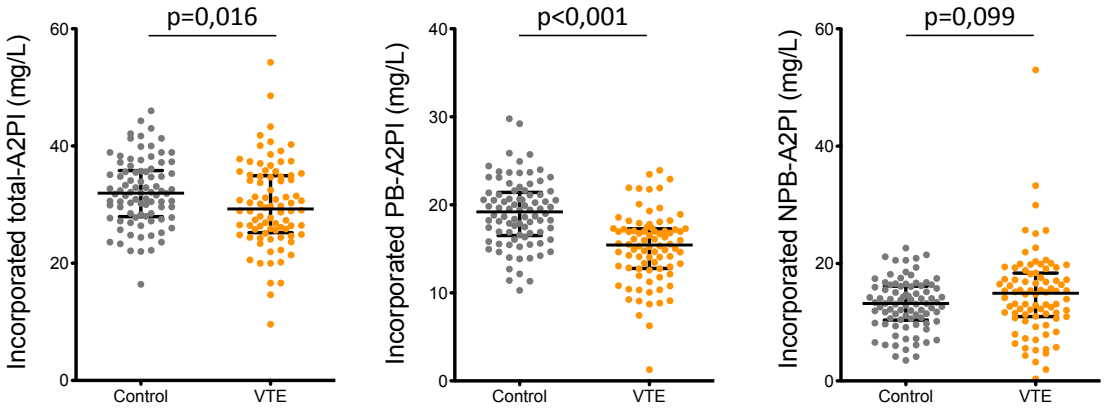

Figure 4. Incorporation of different α2-PI forms into the plasma clot.

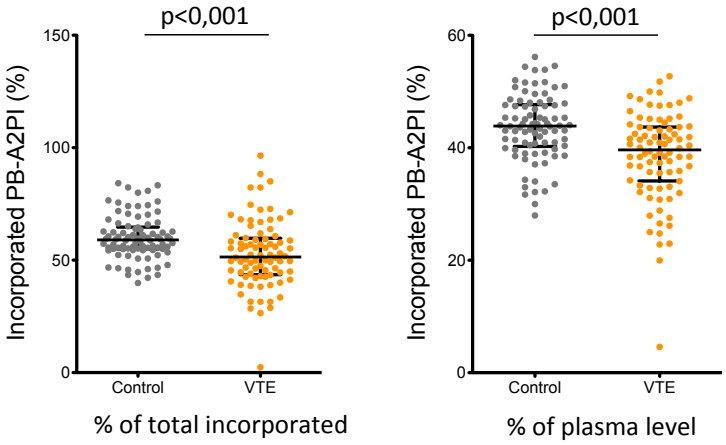

Figure 5. Incorporation of PB- α2-PI into plasma clots. The amount of incorporated PB- α2-PI is expressed as the percentage of total incorporated α2-PI or the percentage of its original plasma concentration.

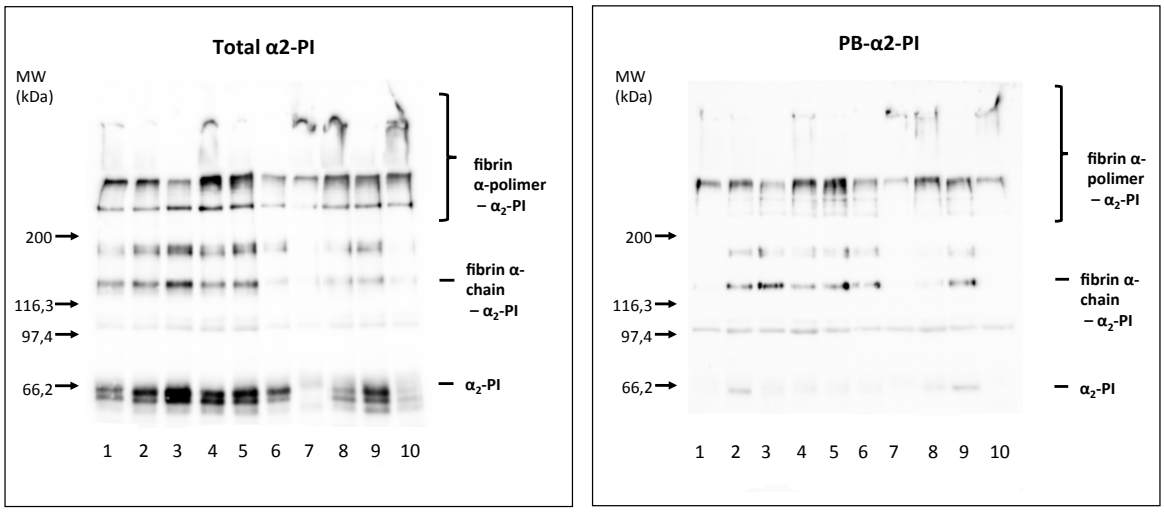

Figure 6. Western blot analysis of washed and dissolved plasma clots prepared from 10 normal plasma samples. Polyclonal anti-α2-PI antibody and anti-PB-α2-PI monoclonal antibody were used to detect total and PB-α2-PI, respectively. MW markers and the position of bands containing non cross-linked and cross-linked α2-PI are marked.

The results suggests that crosslinking of PB-α2-PI – the most active α2-PI form - by FXIIIa to fibrin might be modified by the elevation of NPB-α2-PI concentration in plasma. Moreover, non-covalently attached NPB-α2-PI might also modify clot lysis. However, this hypothesis should be further investigated.

These results were presented as a poster at the ISTH 2021 Virtual Congress and at the 60th Virtual Congress of the Hungarian Society of Laboratory Medicine.

B) To confirm and visualize the binding of C-terminally intact and truncated α2-PI forms to the fibrin network we clotted normal citrated plasma samples by thrombin and Ca^{2+} in IBIDI chambers. After excessive washing of the plasma clot different forms of α2-PI were stained using fluorescently labelled specific antibodies (FITC labelled anti-total α2-PI monoclonal antibody produced by us and Alexa Fluor 647 labeled anti-PB-α2-PI monoclonal antibody). Multicolour STED images were acquired with a Leica TCS SP8 using continuous-wave STED (Stimulated Emission Depletion) gated nanoscopy (Leica Microsystem Mannheim, Germany). Gated STED images were deconvolved using Huygens Professional (Scientific Volume Imaging B.V., Hilversum, Netherlands) software.Image J software was used to investigate the intensity of staining for total and PB-α2-PI. Staining for α2-PI alone makes the fibrin fibers well visible. Bound α2-PI is evenly distributed on the surface of fibrin fibers. The results showed that the NPB-α2-PI form also binds to the fibrin chains as the proportion of the PB-α2-PI form within the total bound α2-PI was 59±7% (n=6), this value is similar to the ratio calculated by ELISA measurements. Fig 7. shows a representative picture of stained plasma clot.

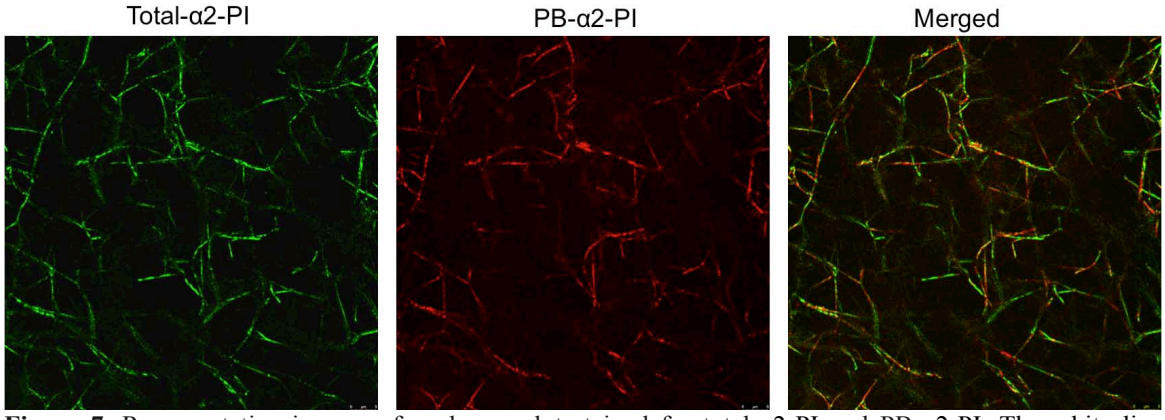

Figure 7. Representative images of a plasma clot stained for total-α2-PI and PB-α2-PI. The white line represents 10 μm.

3.2. Investigation of the amount of α2-PI in coronary thrombus

We aimed to investigate if differences could be demonstrated in the amount of α2-PI present in the thrombus according to the age of the thrombus.
Thrombi were collected by manual thrombectomy aspiration device from patients underwent primary coronary intervention and thrombus aspiration due to the symptoms of acute MI. Median pain-to-balloon time was 8 hours (2-168 hours).

One part of the thrombus was fixed and embedded into paraffin. This was used for standard morphological analysis by which thrombi were categorized into fresh (pain-to-balloon time less that 8 hours), organizing (lytic) (8-24 hours) and organized (older than 24 hours). Another part of the thrombus immediately after aspirating was deep frozen in liquid nitrogen until immuno-fluorescent staining. We established the protocols for multiple immunofluorescent staining and confocal laser scanning microscopy analysis. The staining circumstances were optimized for α2-PI, fibrinogen and FXIII.
All thrombi specimens were stained using the following antibodies: mouse monoclonal anti-total-α2-PI antibody conjugated with FITC (in-house developed), polyclonal rabbit anti-fibrinogen antibody (ab34269, Abcam) used with Alexa Fluor 568 labeled goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, mouse monoclonal anti-FXIII antibody conjugated with Alexa Fluor® 647, (in-house developed). All sections were coverslipped with Hydromount water-based mounting medium (NAT1324, National Diagnostics).
Aspirated thrombi retrieved from 24 male patients were included in the analysis (n=8 for all age group; fresh lytic, and organized, respectively). The image analysis data derived at least three independent experiments from all patients.
As the first step of the evolution of image analysis the multi-area time-lapse software module has been used. Images were acquired by an Olympus FluoView 3000 confocal microscope (Olympus, Tokyo, Japan). This type of module controlled by the Olympus FluoView 3000 confocal microscope's motorized XY stage for multi-dimensional and multi-area time-lapse imaging. Mosaic stitching allows us to take a series of overlapping images and merge them together into a seamless picture of the entire subject, so in this way, tiled images can be enlarged in sections without resolution loss. That was one of the most important steps to observe objectively the degree of heterogeneity in coronary thrombi samples. Quantification of the different characterized elements was done by a standardized methodology. During this phase we determined the mean fluorescence intensity (MFI) of the molecule of interest in a coronary thrombi section using an open-source image analysis tool, Fiji. 1. The mean fluorescence intensity and colocalization with fibrinogen of α2-PI was significantly higher in lytic thrombi compared to fresh thrombi and did not changed significantly in organized thrombi (Fig 8.), suggesting that incorporation of α2-PI into the thrombus lasts for hours. The intensity of fibrinogen showed a continuous elevation and the staining for FXIII did not show significant differences between different age groups. Representative pictures are shown on Fig 9.

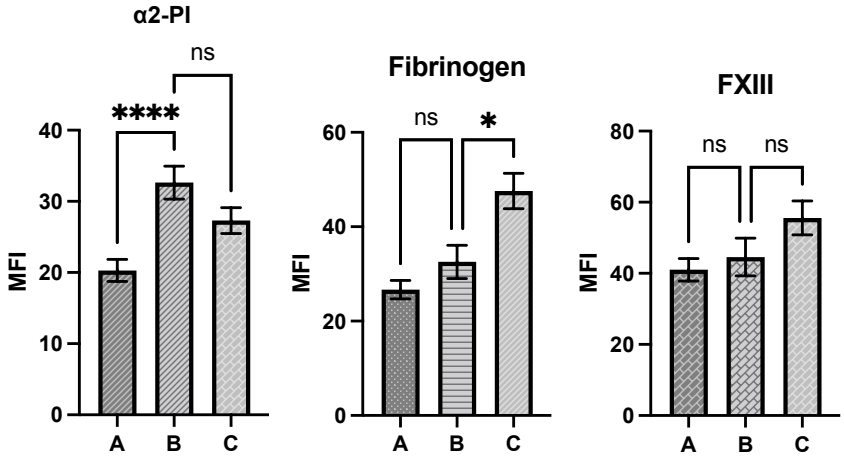

Figure 8. Mean fluorescence intensity values of thrombi with different age: A, fresh; B, lytic; C, organized. *, $p<0.05$; ****, $p<0.0001$; ns, not significant

The results of the colocalization analysis as implemented by the JaCoP plugin in Fiji presented in table 1. Statistically significant difference was found in the case of fibrinogen-α2-PI comparing the fresh and lytic phase. The fibrinogen-FXIII colocalization described a strong correlation in all three examined groups.

Table 1. The results of colocalization analysis (Pearson correlation coefficient (mean values ± SEM, and min/max))

	Fresh thrombus	Lytic thrombus	Organized thrombus
Fibrinogen-α2-PI	0.47±0.02 (0.34/0.66)	0.55±0.02 (0.29/0.67)	0.48±0.02 (0.3/0.69)
Fibrinogen-FXIII	0.64±0.01 (0.51/0.71)	0.65±0.02 (0.47/0.77)	0.67±0.02 (0.44/0.83)

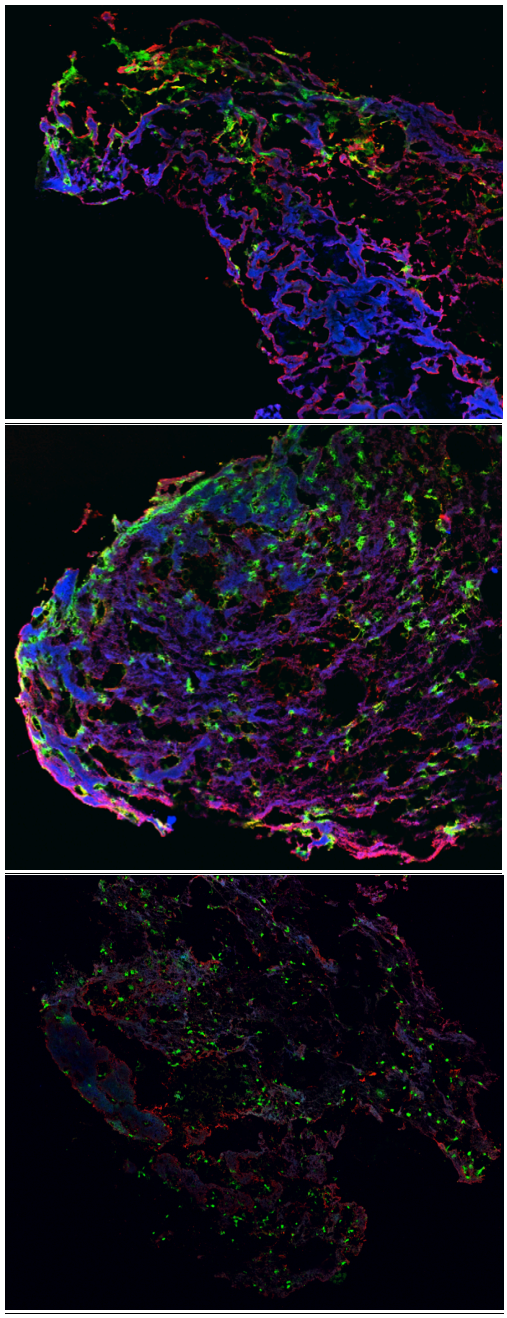

Figure 9. Immunofluorescent staining of thrombi: A, fresh; B, lytic; C, organized. Fibrinogen appears inblue, FXIII in red and α2-PI in green.

3.3. Effect of total α2-PI antigen level and p.Arg6Trp polymorphism on the risk of myocardial infarction in young individuals

One hundred and ten young patients (under 45 years) suffering from acute myocardial infarction (MI) and their matched clinical controls (CC) were enrolled in the study. CC patients had <50% coronary artery stenosis (proved by angiography) and had no history of myocardial infarction. Serum cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, apoAI, apoB, lipoprotein (a), homocysteine, folic acid, vitamin B12, CRP, and plasma fibrinogen, total α2-PI antigen, α2-PI activity, FXIII A_2B_2 and FXIII-B antigen, FXIII activity were measured, and α2-PI Arg6Trp, FXIII-A Val34Leu, FXIII-B His95Arg and FXIII-B Intron K genotypes were determined. The results of 182 apparently healthy controls (HC), under the age of 45 years, were also included in the analysis.
The main results:

- Trp allele frequency did not differ significantly among the study groups and were in good agreement with data obtained from the HapMap database.
- The presence of Trp allele did not influence the risk for MI when patient groups were compared to the CC or HC groups.
- Adjusted total a α2-PI antigen levels (mean (95%CI)) were significantly elevated in MI patients compared to both controls (MI: 75.4 mg/L (73.6-77.1), CC: 72.5 mg/L (70.6-74.3), and HC, 64.0 mg/L (62.5-65.5).
- Individuals having α2-PI levels in the upper third (above 73.3 mg/L) have increased risk for MI (OR: 7.25, 95%CI, 3.53-14,87) than individuals having α2-PI levels below this value.

The results were presented at the 61st Annual Meeting of the Society of Thrombosis and Haemostasis Research, Basel, Switzerland, 15-18 February 2017. (Éva Katona, Adrienn Orosz, Zoltán Mezei, László Balogh, Zsuzsanna Bereczky, István Édes, László Muszbek. Effect of alpha-2 plasmin inhibitor p.Arg6Trp polymorphism and antigen level on the risk of myocardial infarction in young patients)

3.4. Identification of the cleavage site and proteolytic enzyme for the C-terminal truncation of alpha2-plasmin inhibitor (A2-PI)

The C-terminal cleavage site was identified and presented by a Dutch working group (Abdul S et al. On the localization of the cleavage site in human alpha-2-antiplasmin, involved in the generation of the non-plasminogen binding form. Thromb Haemost. 2020 May;18(5):1162-1170. doi: 10.1111/jth.14761.), therefore we started to identify the proteolytic enzyme responsible for the cleavage. Stable transfection of Human embryonic kidney (HEK-293) cells with huSERPIN F2_pcDNA3.1 plasmids was performed with Lipofectamine®3000 Transfection Kit. Geneticin® Selective Antibiotic was used as a selective agent. Cells were grown at 37 °C and 5% CO2 in a humidified incubator. The expression of full length A2PI was verified in the cell media and cell lysate by ELISA and Western blot methods. L-Photo-Leucine and L-Photo-Methionine were used in the culture media to substitute the respective natural amino acids in the expressed α2-PI in order to perform crosslinking reactions with the possible interaction partners. The expressed photo-reactive α2-PI was immuno-precipitated using our monoclonal antibody covalently coupled to Sepharose 4B gel and incubated in fresh human plasma anticoagulated with heparin or EDTA while using UV irradiation from 320 to 370 nm for photoactivation. The identification of cross-linked α2-PI products by SDS-PAGE and Western blot is still in progress. The interaction partners in the cross-linked bands will be identified by mass spectrometric analysis.

Other published results in which the OTKA grant has been acknowledged

We regularly participate in the characterization of inherited and acquired FXIII deficiencies by analysing Hungarian and foreign samples as well and in international FXIII standardization projects. In these projects we apply the methods developed by us for the determination of plasma FXIII and its subunits. We used resources supported by this grant to accomplish those projects. We would appreciate if these publications would be considered as a result of this grant.

In this period we published two research articles characterizing of an autoimmune FXIII deficiency and two new FXIII-A subunit deficiency cases. Two invited articles were published in the Special Issue of ECAT Foundation dealing with the characterization of inherited and acquired FXIII deficiencies. We published a review article, which summarizes auto- and alloantibodies against FXIII subunits published. In addition, we published a research article investigating FXIII levels and FXIII-B subunit polymorphisms in patients with VTE.

1. Bovet et al. Autoimmune factor XIII deficiency with unusual laboratory and clinical phenotype, JOURNAL OF THROMBOSIS AND HAEMOSTASIS 18: (6) pp. 1330-1334., 2020.
2. Plamenova et al. Genetic background of inherited factor XIII-A subunit deficiency: Review of the literature and description of two new cases, Semin Thromb Hemost, DOI https://doi.org/ 10.1055/s-0041-1725170, 2021)
3. Katona É, Muszbek L.: *The laboratory diagnosis of inherited FXIII deficiencies and the measurement of FXIII activity and antigen level*, ECAT Foundation, Special Issue 7: 22-26, 2018
4. Muszbek L, Katona É, Pénzes K: *Antibodies against factor XIII subunits*, ECAT Foundation, Special Issue 7: 17-21, 2018
5. Muszbek L, Pénzes K, Katona É: *Auto- and alloantibodies against factor XIII: laboratory diagnosis and clinical consequences*, Journal of Thrombosis and Haemostasis 16: 822-832, 2018
6. Mezei ZA, Katona É, Kállai J, Bereczky Z, Somodi L, Molnár É, Kovács B, Miklós T, Ajzner É, Muszbek L: *Factor XIII levels and factor XIII B subunit polymorphisms in patients with venous thromboembolism*, THROMB RES 158: 93-97, 2017