NKFIH K 109783
Final report

Major aim of the project was to investigate the effect of α_2-plasmin inhibitor (α_2-PI) plasma level, the ratio of N-terminal α_2-PI variants in the plasma and the α_2-PI Arg6Trp polymorphism on the risk of myocardial infarction. The amount of α_2-PI attached to circulating fibrinogen was also examined.

Development of sandwich type immunoassay for the measurement of N-terminally intact α_2-PI (Met1-α_2-PI) using newly generated monoclonal and commercially available polyclonal antibodies.

We started the work with generation of monoclonal antibodies against the peptide corresponding to the N-terminal twelve amino acids of Met1-α_2-PI (Fig 1., P1-12). We fused Sp-2 myeloma cells and spleen cells of mice immunized with the peptide-KLH conjugate. Antibody production was tested by indirect ELISA systems, in which peptide conjugated to bovine serum albumin, un-conjugated peptide or native α_2-PI were coated onto the surface of the ELISA plate. Seven antibody-producing hybrid cell lines were selected and cloned which produced antibody that bound equally well to the peptide and the native α_2-PI protein isolated from human plasma. Antibodies were purified from ascites fluid by Protein G affinity chromatography and tested in sandwich type ELISA assay as capture antibody, while HRPO labelled polyclonal anti-α_2-PI was used as tag antibody. The monoclonal antibody 3/2D6F4 was selected for developing a sandwich type ELISA to measure Met1-α_2-PI in human plasma. Unfortunately the assay measured much lower level of Met1-α_2-PI in normal plasma samples than expected and it turned out, that the *p.Arg6Trp* polymorphism of α_2-PI influenced the antigen-antibody reaction. None of the selected antibodies reacted with α_2-PI(Trp6). In order to overcome the problem caused by the effect of R6W polymorphism we chose an alternative way to produce antibodies that bind specifically to the N-terminally intact α_2-PI, irrespective of the polymorphism. We synthesized a new peptide that does not contain the polymorphic site, but contains the APCE cleaving site (Fig 1., P8-20).

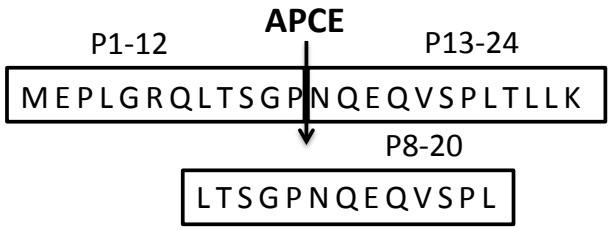

Fig 1. Peptides corresponding to the N-terminal end of α_2-PI

The peptide was conjugated to KLH for immunization and BSA for testing of antibody production of hybrid cells. Mice were successfully immunised with the new peptide-KLH conjugate and the protocol of monoclonal antibody production was repeated several time. We were able to select an antibody that can bind only the Leu8-Leu20 peptide and intact recombinant Met1-α_2-PI protein and does not react with the P1-12 or P13-24 peptides, which means that the epitope of the antibody is lost when APCE cleaves off the N-terminal twelve amino acids from Met1α_2-PI. The antibody

was purified by Protein G affinity chromatography and conjugated to horseradish peroxidase enzyme. We wanted to develop a sandwich type immunoassay using this antibody and a second antibody that can bind all α_2-PI isoforms equally well. However, the sandwich system did not work with plasma samples. We repeated the antibody generating protocol several times in order to find a suitable antibody, but did not succeed. Therefore, we changed our strategy again; we immunized mice with purified native α_2-PI protein and the specificity of the generated antibodies were tested by investigating their binding to native α_2-PI and to different peptides corresponding to the N-terminal end of α_2-PI (Fig 1.). This approach is generally thought to be less effective to get antibody to a specific part of the antigen, but in our case it seems to be more successful. We identified several positive hybrid cultures with different epitope specificities (Table 1.)

Table 1. Reaction of newly generated antibodies with purified α_2-PI and its N-terminal peptides

Hybridoma code	**OD value in different indirect ELISAs**		
	α_2-PI	**P1-12**	**P8-20**
1A1	3.27	0	0
1B1	1.03	0	0
1B4	1.34	1.36	0.24
1C2	0.79	0	0
1C3	1.21	0.10	0
1D2	1.10	0	0
1F1	2.16	0.44	0
1F4	1.20	0	0.73
2A3	1.52	0	0
2B3	3.51	0	0
2E3	1.57	0	0

The reaction pattern of antibodies 1B4 and 1F4 is promising. After cloning the hybrid lines the epitope specificity of antibodies will be tested again and their reaction (as capture antibody) with plasma α_2-PI in sandwich type ELISA will also be investigated. Antibodies reacting with other parts of the α_2-PI molecule will also be tested as tag antibody in the sandwich ELISA system. The continuation of the work is planned within the framework of the ongoing project of principal investigator "The role of alpha2-plasmin inhibitor natural heterogeneity in thrombotic diseases" (NKFI K120633).

Determination of the ratio of N-terminal α_2-PI variants in normal plasma samples, and its correlation with the α_2-PI R6W genotype.

α_2-PI *p.Arg6Trp* genotype was determined in DNA samples of 265 apparently healthy volunteers (age: 18-70 years). The frequency of Arg6 and Trp6 alleles was 0.779 and 0.221, respectively. Our results well agreed with the results published earlier (Arg6: 0.795, Trp6: 0.205; Christiansen et al. Blood, 2007;109:5286-92.).

Total α_2-PI antigen and activity levels were measured from 221 samples using the ELISA method developed in our laboratory and Berichrom α_2-Antiplasmin kit,

respectively. Fibrinogen, FXIII activity, FXIII-A_2B_2 and FXIII-B antigen levels were also determined.

Results: α_2-PI antigen and activity results did not show Gaussian distribution, the median (min, max) plasma concentration and activity were 63.2 (34, 87,5) mg/L and 120 (46, 150) % respectively. There were no gender differences in the antigen and activity levels and age also was without effect. Total α_2-PI antigen showed significant correlation with the activity levels (r=0.591, p<0,001) and moderate, but statistically significant correlation with fibrinogen (r=0,291, p<0,001) and FXIII-B antigen (r=0,241, p<0,001). α_2-PI *p.Arg6Trp* genotype had no significant effect on total α_2-PI antigen and activity levels.

Plasma aliquots are stored for the determination of Met1α_2-PI isoform using the method that is under development.

α_2-PI R6W genotype frequency in the general Hungarian population.

α_2-PI R6W genotype was determined in DNA samples of 999 individuals representing the general Hungarian population. Individuals were recruited in the framework of the Hungarian General Practitioners' Morbidity Sentinel Stations Program. The frequency of R and W alleles was 0.785 and 0.215, respectively.

α_2-PI Arg6 allele frequencies in the general population and in the healthy control group did not differ significantly.

Effect of α_2-PI R6W genotype on the risk of myocardial infarction.

α_2-PI R6W genotype was determined in stored DNA samples of the CAD study group investigated earlier in our laboratory. The study group included 750 consecutive patients admitted for coronary angiography to investigate suspected coronary artery disease. Patients were recruited for the study from a single centre (Institute of Cardiology, University of Debrecen, Debrecen, Hungary) over a one and a half year period. Patients with ≥50% stenosis in a major coronary artery or in one of their branches were graded as coronary sclerosis positive (CS+), while patients with no or less significant stenosis were graded as CS-. Patients with positive or negative history of MI were classified as MI+ or MI-, respectively. Patients without significant coronary stenosis and with the lack of history of MI were considered as the clinical control group (CS-MI-) to which subgroups of patients with CS and/or MI (CS-MI+, CS+MI-, CS+MI+) were compared. Table 2 shows the α_2-PI R6W genotype distribution in the different patient groups.

Table 2. α_2-PI R6W genotype distributions in the population control and CAD patient groups. Results are presented as N (%)

Allele	Population control	Clinical control	CS-MI+	CS+MI-	CS+MI+
CC	625 (62.6)	171 (66.0)	18 (64.3)	146 (62.4)	151 (65.9)
CT	318 (31.8)	75 (28.9)	7 (25.0)	76 (32.5)	70 (30.6)
TT	56 (5.6)	13 (5.0)	3 (10.7)	12 (5.1)	8 (3.5)
Total number	999	259	28	234	229

Main results of the study:

- α2-PI Arg6Trp genotype distribution did not differ significantly among the different control and patient groups (Table 1.)

• The presence of Trp allele did not influence the risk of CAS or MI when patient groups were compared to the clinical control or population control groups. Homozygous Trp6 genotype conferred a decreased, but statistically not significant risk of MI (Table 3.)

Table 3. Effect of α2-PI Arg6Trp polymorphism on the risk of CAS or MI (OR (95% CI); p)

Control group	**Genotype**	**CAS+MI-**	**CAS+MI+**
Population control	OR for Trp6 carriers	1,007 (0,751-1,351); 0,962	0,863 (0,638-1,167); 0,340
Population control	OR for Trp6 homozygotes	0,917 (0,479-1,755); 0,794	0,591 (0,276-1,267); 0,176
Clinical control	OR for Trp6 carriers*	1,109 (0,740-1,661); 0,616	0,972 (0,635-1,487); 0,896
Clinical control	OR for Trp6 homozygotes*	0,908 (0,368-2,239); 0,834	0,539 (0,192-1,513); 0,240

The results were presented at the XXV Congress of the International Society on Thrombosis and Haemostasis, Toronto, Canada, 20-25. June 2015.

Effect of α_2-PI R6W genotype on the risk of myocardial infarction in young patients

116 patients who had coronary sclerosis proven by coronary angiography and had ST elevation myocardial infarction (MI) below the age of 40 and 120 healthy controls (HC) were enrolled in this part of the study. 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 main results:

Table 4. Main characteristics of the patients groups

	HC (n=120)	**MI (n=116)**	p
Age (median (min – max)	31.5 (24-40)	36 (14-40)	<0.001
Male/female (n)	58/62	91/25	<0.001
Diabetes mellitus (%)	0	14	<0.001
Smoking (never/current/ex) (%)	60/28/12	19/54/27	<0.001
Positive family history (%)	38	72	<0.001
Hypertension (%)	11	43	<0.001
Hyperlipidemia (%)	ND	87	NA
BMI (mean±SD)	24.5±4.2	28.4±5.4	<0.001
Fibrinogen g/L (mean±SD)	3.19±0.60	3.65±0.77	<0.001
α2-PI Arg6Trp n (%)			
RR	77 (64.2)	72 (62.1)	

RW	39 (32.5)	36 (31.0)	
WW	4 (3.3)	8 (6.9)	
W carrier frequency (%)	35.8	37.9	0.788
MAF (%)	0.20	0.22	

Positive family history was defined as MI, stroke, or non-traumatic lower limb amputation in first-degree relatives; Hypertension: systolic blood pressure ≥ 140 mmHg and/or diastolic pressure ≥ 90 mmHg or the patient was on anti-hypertension medication prior admission; Hyperlipidemia: LDL-cholesterol level >3.4 mmol/l or the patient was on cholesterol lowering medication prior admission; ND: not determined; NA: non applicable

- Trp allele frequency did not differ significantly among the study groups and were in good agreement with data obtained in the CAD study group and from the HapMap database.
- The presence of the Trp allele did not influence the risk for MI; OR (95% CI; p) was 1.104 (0.595-2.046; 0.755).
- α2-PI antigen levels adjusted to gender and fibrinogen (mean (95%CI)) were significantly elevated in MI patients compared to controls (MI: 74.32 mg/L (73.6-77.1), HC, 64.0 mg/L (62.5-65.5).
- α2-PI levels in the upper tertile (above 73.3 mg/L) increased the risk of MI (OR MI vs HC, 7.25, 95%CI, 3.53-14,87).

Results were presented at the 61st Annual Meeting of the Society of Thrombosis and Haemostasis Research, Basel, Switzerland, 15-18 February 2017. and at the XXVI ISTH Congress and 63rd Annual SSC Meeting, Berlin, Germany, July 8-13 2017. An MSc student participated in the measurement of total α2-PI antigen and activity levels, therefore part of the results were presented in a diploma-thesis. The manuscript is under preparation.

Effect of factor XIII levels and polymorphisms on the risk of myocardial infarction in young patients

In the young patient cohort we also investigated the association of FXIII levels with the risk of myocardial infarction. FXIII activity and FXIII-A_2B_2 antigen levels were significantly elevated in MI. Smoking had an independent increasing effect on FXIII activity and FXIII-A_2B_2 antigen. The effect of smoking on coronary thrombus formation may partially be attributed to its FXIII increasing effect (Balogh L, Katona É, Mezei ZA et al. Effect of factor XIII levels and polymorphisms on the risk of myocardial infarction in young patients. Molecular and Cellular Biochemistry 2018;448:199-209.).

The amount of covalently cross-linked α2-PI in circulating fibrinogen.

To investigate the amount of α_2-PI covalently attached to circulating fibrinogen monoclonal anti-α_2-PI antibody that binds all plasmatic form of α_2-PI (developed in our laboratory) was coupled covalently to Sepharose 4B gel and used for the immun-precipitation of α_2-PI from citrated normal human plasma samples. After washing of the gel, the precipitated α_2-PI and other molecules that bound to it were analysed by two different techniques.

1/ Immuno-precipitated proteins were eluted by SDS-PAGE sample buffer that was followed by the addition of 2-mercaptoethanol to reduce the proteins. Reduced

proteins were separated by SDS-PAGE (two parallel gels), electro-blotted to PVDF membranes and analysed by Western blotting technique using polyclonal anti-α_2-PI or polyclonal anti-fibrinogen antibodies and ECL detection system. By this technique we detected only isolated fibrinogen subunits or α_2-PI with 67 kD molecular weight and high molecular weight cross-linked complexes were not seen either of the Western blot membrane. From these results it seems that in contrary to the published results α_2-PI in the circulation doesn't binds covalently to fibrinogen. Western blots were analysed by quantitative densitometry and 0.07-0.8 nM fibrinogen/1 nM α_2-PI was detected in the precipitates. The reproducibility of the method however was not acceptable.

2/ To determine the amount of non-covalently attached α_2-PI-fibrinogen complex in the plasma accurately we developed an in-house fibrinogen ELISA assay and modified the immuno-precipitation protocol. Precipitation was performed in compact reaction columns developed for small-scale affinity purification, which provides effective washing of the gel. Proteins bound to monoclonal anti-α_2-PI-Sepharose 4B gel were eluted by glycine buffer (pH: 2.7) and after neutralizing the pH fibrinogen and α_2-PI were measured by ELISA methods developed in our laboratory. In the precipitate of 20 healthy volunteers 11.8±1.1 μg/ml (176±17 nM) α_2-PI and 353±408 ng/ml (1.04±1.2 nM) fibrinogen was detected, which means that α_2-PI in the circulation is either not bound to fibrinogen or the affinity is very low.

The effect of fibrin monomer formation on the binding of α_2-PI was also investigated by this technique. We added Ca^{2+} and thrombin to normal human plasma samples in order to cleave off the fibrinopeptide A and B while GPRP peptide and IAA were present to inhibit fibrin polymerization and FXIII activity, respectively. The amount of fibrin(ogen) in the eluate was increased by ≈10 times after fibrin monomer formation.

The results were verified using appropriate negative controls. Using normal mouse IgG covalently attached to Sepharose 4B for the immuno-precipitation of plasma samples and applying different surface blocking techniques we confirmed that fibrinogen measured in the eluates of anti-α_2-PI-Sepharose 4B gel were precipitated together with α_2-PI and was not bound to the gel by nonspecific binding.

Part of the results was presented at the congress of Hungarian Society on Thrombosis and Haemostasis (2014). The manuscript is under preparation.