Hereby I report the results of the three-years research supported by OTKA (PD101120) following the same order as it was given in the original Workplan.

I. Laboratory diagnosis of AT/PC/PS deficiencies

During the three-years period enrollment of patients with low activity of Antithrombin (AT), Protein C (PC) or Protein S (PS) measured by the routinely (and widely) used tests were carried out. All the hemostasis laboratory investigations and molecular genetic analysis were performed. By the end of the research period altogether 142 patients having AT deficiency, 180 patients with low PC activity and 166 patients with low PS activity in the respective functional clotting assays were recruited.

I/1Antithrombin (AT) deficiency:

AT deficiency is screened by functional amidolytic assay in the presence of heparin, which is named as heparin cofactor AT activity. According to the international guidelines, this test can be performed either in the presence of thrombin or activated FX. In the former assay the thrombin inhibition effect (anti-FIIa activity) of AT is detected, while in the latter test the activated FX inhibition effect (anti-FXa activity) of AT is measured. The two types of assay are equally used worldwide as first line tests in the diagnosis of AT deficiency. At the beginning of our research, we have demonstrated and reported that the heparin cofactor anti-FXa (hc-anti-FXa) assay is superior over the thrombin inhibition assay in detecting type II-heparin binding site (II HBS) AT deficiency *(Am J Clin Pathol 2013;140:675-679)*.

Briefly, 37 consecutively diagnosed patients with AT deficiency, proved by fluorescent DNA sequencing, were recruited for the study. Heparin cofactor AT activity was determined by measuring the inhibition of FIIa or FXa by the patient's plasma. Dade Behring Berichrom antithrombin III test (Marburg, Germany) was used to determine anti-FIIa activity; the reagent kit includes bovine thrombin and tosyl-Gly-Pro-Arg-5-amino-2-nitrobensoic acid isopropylamide substrate. Anti-FXa activity was performed with two assays: Siemens (Marburg, Germany) Innovance antithrombin kit (anti-FXa1) and another anti-FXa based method, established by our group (anti-FXa2). The former uses human FXa with benzoylcarbonyl-D-Leu-Gly-Arg-5-amino-2-nitrobensoic acid methylamide acetate substrate, whereas the latter uses FXa of bovine origin and succinyl-Ile-Glu(γPip)Gly-Arg-paranitroaniline HCl substrate. AT antigen was measured by immunonephelometry (BN ProSpec System AT-III, Siemens). Among the recruited patients, seven had type I defect (quantitative AT deficiency), one had type II PE (ie. AT deficiency with pleiotropic effect), and 29 patients from 20 families had type II HBS deficiency (nine carried the mutation in homozygous form, while 20 patients were heterozygotes). The two anti-FXa AT assays gave practically identical results in all AT-deficient patients. Anti-FIIa and anti-FXa results in type I–deficient patients and in the single type II PE patient did not differ significantly; all were uniformly below 80%, the lower limit of the reference interval. In contrast, with a single exception, anti-FIIa activity of type II HBS heterozygotes was in the reference interval (range, 76%-128%), whereas all anti-FXa activities in this group were below the reference interval (range, 55%-73% for the anti-FXa1 method and 46%-74% for the anti-FXa2 method). Figure 1

demonstrates the differences between anti-FIIa and anti-FXa assays in sensitivity to type II HBS AT deficicency.

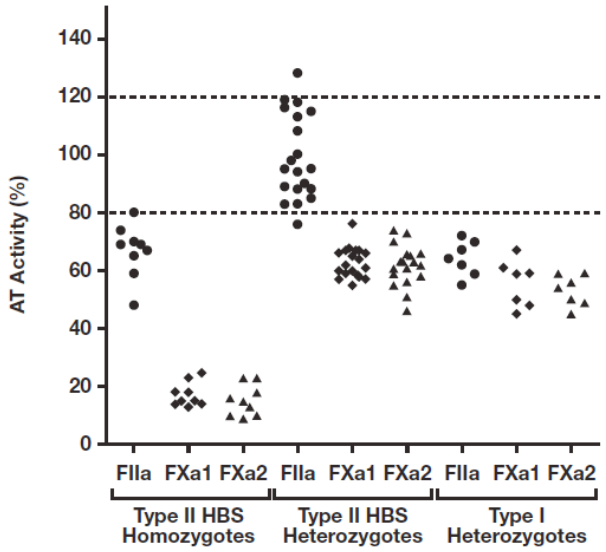

Figure 1. AT activity values in type II HBS deficient patients and in type I (quantitative) AT deficients. The activity values between the two horizontal dashed lines are within the reference interval.

The results of this study suggest that anti-FIIa assays are not able to detect heterozygous type II HBS AT deficiency and might even miss some homozygotes. For this reason, in countries such as Hungary where type II HBS deficiency occurs with high frequency (see later) we recommend the use of an anti-FXa assay as the first-line test.

As it is suggested by former publications the clinical phenotype of type II HBS AT deficiency differs from that of other subtypes. As opposed to other subtypes, its homozygous form is not lethal, although it results in severe VTE at an early age. Heterozygous type II HBS AT deficiency is a less severe phenotype than other heterozygous subtypes. Therefore, it is important to introduce a laboratory assay, which can distinguish between type II HBS AT deficiency and other subtypes. It was assumed that the progressive anti-FXa (p-anti-FXa) activity, measured in the absence of heparin, is insensitive to HBS defect and its parallel measurement with hc-anti-FXa activity provides a tool for the diagnosis of type II-HBS deficiency. Based on this hypothesis we modified the anti-FXa chromogenic AT assay to measure both the hc-anti-FXa and p-anti-FXa activity, evaluated the assays, established their reference intervals and on a relatively high number of AT deficient patients demonstrated its usefulness in the diagnosis of type II-HBS deficiency *(Clin Chem Lab Med 2014; 52:1797-1806).*

Briefly, patient plasma diluted five-fold in EDTA and polybrene containing Tris-HCl buffer is incubated with equal volume of FXa (12 nkat/mL) for 300 sec at 37°C. After incubation 50 **µ**L 1.25 mg/mL BIOPHEN CS-11(32) [Suc-IIe-Gly-(**γ**Pip)Gly-Arg-pNA, HCl] chromogenic substrate (Hyphen BioMed, Neuville sur Oise, France) was added and the release of pNA by uninhibited FXa was recorded at 405 nm for 60 s. The same assay components were used for the determination of hc activity, with the following

exceptions: in this case polybrene was replaced by 1 U/mL heparin in the dilution buffer, the dilution of plasma samples was 50-fold and the incubation with FXa was shortened to 60 s. The **Δ**A/min values were converted to percentage of mean normal anti-FXa activity. In summary, the plasma volume was increased 10-fold and the incubation time was prolonged 5-fold in the p-anti-FXa assay as compared to the hc-anti-FXa assay. The methods were adapted to automated coagulometers (Siemens BCS-XP and Technoclone Ceveron) and evaluated according to the EP15-A2 guideline of Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA). To determine what portion of p-anti-FXa activity was due to FXa inhibitor plasma proteins other than AT, the anti-FXa activities of AT deficient plasma (Enzyme Research Laboratories, Swansea, UK), 2.0 mg/mL purified **α**1-antitrypsin and **α**2- macroglobulin (both from Sigma-Aldrich) were also measured. The p-anti-FXa activity of 2.0 mg/mL **α**1-antitrypsin corresponded to 9.5% of normal plasma p-anti-FXa activity, while **α**2-macroglobulin in the same concentration exhibited only 4.0% inhibitory activity. These findings indicated that AT was responsible for approximately 80% of p-anti-FXa activity present in the plasma. After these methodological considerations the reference intervals for both p-anti-FXa and hc-anti-FXa assays were determined on 188 healthy individuals recruited for this purpose. The reference intervals were established as described in the CLSI EP28-A3c guideline. The distribution of both hc-anti-FXa and p-anti-FXa activities was normal and the reference intervals calculated by the parametric and non-parametric methods gave identical results: in the case of hc-anti-FXa assay they were 82%–118% and 81%–117%, respectively and in the case of p-anti-FXa test they were 82%–118% (parametric method) and 84%–117% (non-parametric method). After method establishment and evaluation the usefulness of simultaneous determination of p-anti-FXa and hc-anti-FXa tests were examined on the plasma samples of seventy-eight AT deficient patients. Among them eight had type I defect, one had type II-PE, and 69 patients had type II-HBS deficiency. In homozygous type II HBS deficient patients the hc-anti-FXa assay gave a very low AT activity result (median 12%; range 8-26%), while the p-anti-FXa assay gave activity values highly overlapping with the reference interval (median 77%; range 64-106%). The AT antigen concentrations (median: 79%; range 51–98%) corresponded to the p-anti-FXa activities. In heterozygotes the decrease of hc-anti-FXa activity corresponded to the heterozygous state (median: 51%; range 34–65%), while the p-anti-FXa activity (median 90%; range: 66–111%) only marginally decreased and there was no overlap between the ranges of the two anti-FXa activities. In contrast to type II HBS patients in the case of type I heterozygotes and in type II-PE heterozygote both anti-FXa activities were below the reference interval. The calculated ratios p/hc ratios for each AT deficient group and healthy persons are shown on Figure 2.

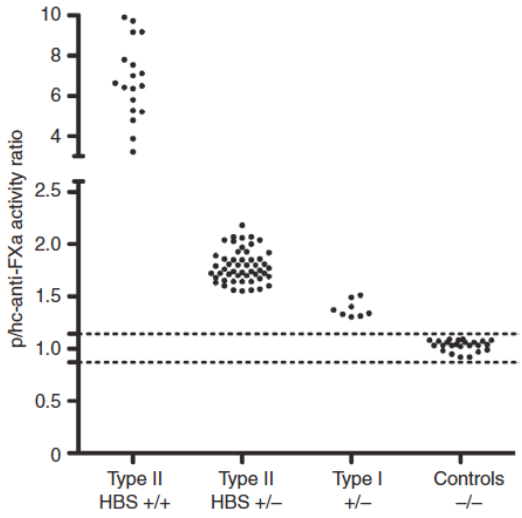

Figure 2. The p-anti-FXa and hc-anti-FXa ratio values in type II HBS homozygotes, heterozygotes, in type I deficient and in healthy controls.

The ratios of p/hc-anti-FXa assays for type II-HBS heterozygotes (range 1.54–2.21) were well above the upper limit of the reference interval (range 0.87–1.14), and could be clearly separated from the group of wild type persons. In the case of homozygous type II-HBS AT deficient patients the ratios were much higher (range 3.23–9.63) than those of heterozygotes. In type I deficient patients the p/hc-anti-FXa ratio was only slightly elevated. As AT antigen is proportionally low in type I deficiency this slight overlap with the type II HBS heterozygous individuals do not influence the diagnosis. In summary, the above results suggested that comparing p-anti-FXa and hc-anti-FXa activities could be a useful tool in the diagnosis of type II HBS AT deficiency and a new laboratory algorithm was established (Figure 3).

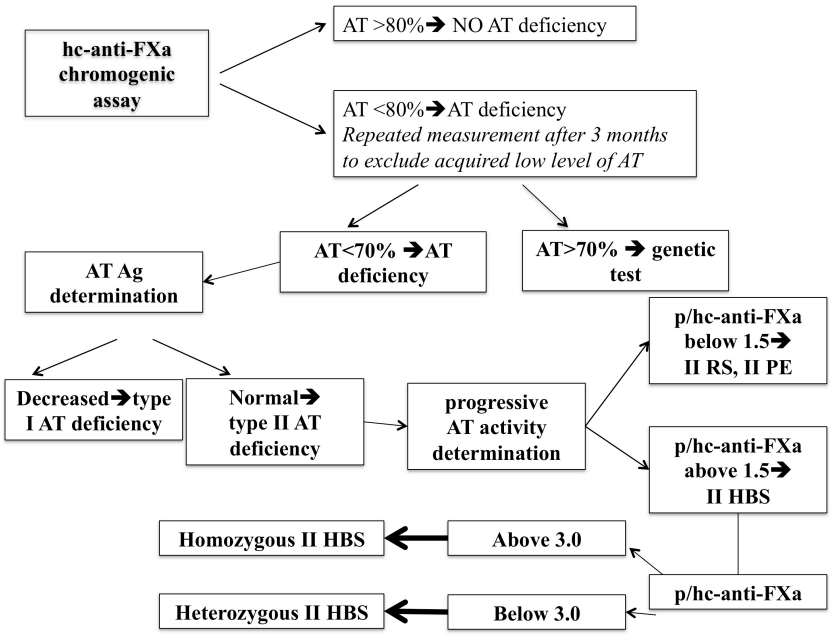

Figure 3. Laboratory diagnostic algorithm for AT deficiency.

AT Budapest 3 (ATBp3, p.Leu99Phe, which is named as p.Leu131Phe according to the HGVS nomenclature) is a missense mutation leading to type II-HBS AT deficiency. In the literature only sporadic data existed concerning this mutation; before us no population-based studies were conducted. In this study, our aims were to determine the frequency of ATBp3 in Hungarian AT deficient patients and examine the possibility of a founder effect, since we detected a high number of patients carrying this mutation in our previous work. In the study period 142 AT deficient patients were diagnosed and the mutations were detected by direct DNA sequencing. Among them 72% had ATBp3 mutation. Beside general personal data, clinical conditions as type of VT (i.e. deep vein thrombosis (DVT), pulmonary embolism (PE) or both) diagnosed by objective imaging methods, site of localization, age at the first thrombotic event, number of further thrombotic events (arterial or venous), presence of provoking factors at the time of VT onset, presence of positive family history of VT, presence of acquired risk factors and therapeutic modality were collected. A large number of individuals (n=1000) representing the general Hungarian population were recruited in the framework of the Hungarian General Practitioners' Morbidity Sentinel Stations Program (HMSSP) and served as population controls (PC) for the study. First, we established a fast method, a real-time PCR followed by melting curve analysis on a LightCycler 480 instrument (Roche, Mannheim, Germany) using oligonucleotide primers and fluorescently labeled probes designed by us. The primers were as follows, forward - *5'-GGCAGATTCCAAGAATGACA-3'*, reverse - *5'-TGAGGAATCATTGGACTTGG-3'*, anchor-*5'-LCRed610-AGGCACCCAGCTTGGTCATA–Phosphate-3'*, sensor - *5'-CAGTTGCTGGAGGGTGTCATTA-Fluorescein-3'*. Annealing temperature was 53°C, amplification was performed by 45 cycles. Determination of ATBp3 was executed in the

PC group (n=1000) and no ATBp3 mutation was found among them. This suggests that the occurrence of ATBp3 is less than 1:1000 in the general Hungarian population.

To investigate the founder effect ATBp3 patients, relatives and randomly selected individuals from the PC group (n=200) were genotyped for five polymorphic markers in SERPINC1 gene, and four microsatellite markers in and around SERPINC1 (Figure 4).

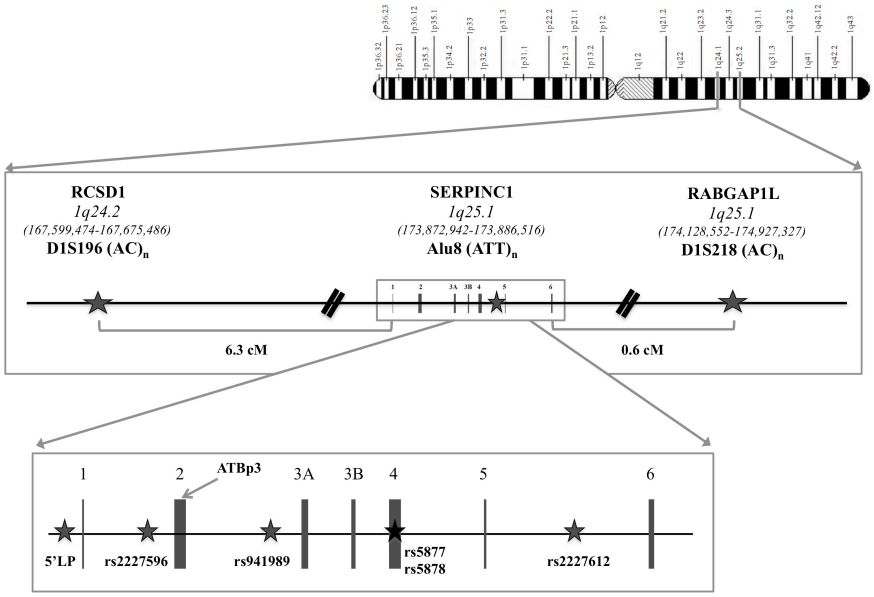

Figure 4. Polymorphic markers and SNP's in and around SERPINC1 used in the study. Short tandem repeat markers are Alu8 (ATT)n, which locates within intron 5 of SERPINC1, D1S196 $(AG)_n$ locates 6.3 cM proximal to SERPINC1 and D1S218 $(AG)_n$ locates 0.6 cM distal to SERPINC1. 5' length polymorphism and the examined single nucleotide polymorphisms locate within SERPINC1. Vertical lines represent exons and the width of them is proportional to the respective exon size.

The 5'length polymorphism (5'LP), which is a 32 (frequency in Caucasians 0.79) or 108 bp (frequency in Caucasians 0.21) non-homologous sequence, locates 345 bp upstream the translation initiation codon of SERPINC1. PCR-RFLP method was introduced to detect this variant. Two silent single nucleotide polymorphisms (SNP), rs5877 (A>G, p.Val327, minor allele frequency, MAF in Caucasians 0.326), and rs5878 (A>G, p.Gln337, MAF in Caucasians 0.332) within exon 4 were determined by direct fluorescent sequencing on ABI-3130 Genetic Analyzer and analyzed by Sequencing Analysis 5.1.1 software (Life Technologies, Carlsbad, CA). Analysis of three additional SNPs within intronic regions of SERPINC1, rs2227596 (A>G, nt1725, MAF in Caucasians 0.205), rs941989 (G>A, nt4647, MAF in Caucasians 0.332), rs2227612 (A>C, nt11117, MAF in Caucasians 0.133) was executed by real-time PCR, using melting curve analysis on a LightCycler480 Instrument. A method for the analysis of four microsatellite markers (chromosome 1 SERPINC1-Alu8-ATTn, D1S196 GTn, D1S218

GTn and as a negative control chromosome 6 F13A1 AAAGn) was also established using multiplex PCR followed by fragment analysis on an ABI 3130 Genetic Analyzer (Table 1). Analysis of these short tandem repeat (STR) sequences was performed by GeneMapper v4.1 software (Life Technologies).

Genetic markers	Primer	Sequence 5' to 3'	Labeling	T_{an} (°C)	Fragment size (bp)
rs2227596	F	*GTGGAACTGTCCAGCAAATAG*		57	262
	S	*TGCACTCCAACCTGGG**T**GACA*	FL		
	A	*AGTGAGACTGTGTCTCAAAACAGCAA*	LCRed610- PH		
	R	*CTAGTAGCTTGGGACTACAGGTG*			
rs941989	F	*GTACTTCCATATCACTCATGTCTCTG*		57	251
	S	*CAAAAGCTTGGTGCTGAAT**C**TCTCT*	FL		
	A	*TTCTCCAGAGAGATTCAGTCTCACCC*	LCRed670- PH		
	R	*GCCTATTGGAGCAAAATCAG*			
rs2227612	F	*CTCATGGGAGATGAACAGTACACAC*			361
	S	*TAACTACTGCTGTTGGTCCATAGTT**T**AGTGT*	FL		
	A	*TACTTGAGAACCAGAAGTTCCACAGG*	LCRed610- PH		
	R	*TACTTGAGAACCAGAAGTTCCACAGG*			
Alu8	F	AAGCTGAAGCCTGAGAATGAAT	6FAM™	62	138-177
	R	GTTGCAGTGAGCCAAGATCA			
D1S196	F	TTGGGCCCTTATTACATACCAGA	PET®		271-287
	R	GATTTGGATTCGTTACATGTTTCTCTT			
D1S218	F	CTGTTTATGTTATCACCAAGGCTTCT	VIC®		345-365
	R	TCGTGAAATGTAATCCTCATGAATAAC			
F13A1-STR	F	GAGGTTGCACTCCAGCCTTT	NED™	62	211-247
	R	GCCCCAAGGAAGATGAGTAAAC			

Table 1. **P**rimer and probe characteristics of genetic markers used in the study.
F - forward primer; R - reverse primer; A - anchor probe; S - sensor probe; FL – Fluorescein; PH – Phosphate; T_{an} – annealing temperature

Allelic frequency distribution of the above-mentioned genetic markers was determined in ATBp3 carriers and healthy controls, haplotype and family tree analyses were also performed. Minor allele frequency (MAF) in the population control group showed good similarity with the HapMap data for European population for each SNP and for the 5'-length polymorphism. MAF values differed significantly in patients carrying the ATBp3. While ATBp3 was associated with the same SNP haplotype in all carriers ("AASAGA" for rs5877, rs5878, 5'-length polymorphism, rs2227612, rs941989 and rs2227596, respectively), different haplotypes were observed in non-carriers (Table 2).

ATBp3 carriers (n=99)									General population (n=200)						
		rs5877	rs5878	5'LP	rs2227612	rs941989	rs2227596	frequency			5'LP	rs2227612	rs941989	rs2227596	frequency
ATBp3 „T allele"		A	A	S	A	G	A	0.595							
ATBp3 „C allele"	Haplotype 1	A	A	S	A	G	A	0.198	ATBp3 „C allele"	Haplotype 1	S	A	G	A	0.630
	Haplotype 2	G	G	L	A	A	A	0.095		Haplotype 2	L	A	A	A	0.104
	Haplotype 3	G	G	L	C	A	G	0.086		Haplotype 3	L	C	A	G	0.147
	Haplotype 4	G	G	S	A	A	A	0.017		Haplotype 4	S	A	A	A	0.077
										Haplotype 5	L	A	A	G	0.033

*S, short, 32 bp; L, long, 108 bp

Table 2. Results of haplotype analysis in ATBp3 carriers and in the general population.

Concerning the microsatellite analysis ATBp3 homozygous patients shared one distinct Alu8 repeat number variation $(ATT)_{15,}$ while in controls different STR repeat numbers were seen between 1 and 19 and repeat number 14 was the most frequent. The STR marker closer to SERPINC1 showed biallelic distribution in ATBp3 homozygotes carrying $(AC)_{24}$ and $(AC)_{25.}$ The distribution of the population control group was heterogeneous, repeat numbers between $(AC)_{19}$ and $(AC)_{33}$ were observed. STR marker D1S196, which locates proximal and in a higher distance from SERPINC1 showed high degree of variability both in ATBp3 homozygotes and in the control group. Repeat numbers between $(AC)_{11}$ and $(AC)_{18}$ were registered and the most frequent repeat number was 12 in both groups. As a negative control, one extra-chromosomal STR marker (F13A1) was also screened and showed heterogeneity in both the 200 controls and in the ATBp3 patients and $(AAAG)_n$ repeat number was found to be between 3 and 20. Family tree analysis, performed in seven families, also suggested founder effect. Oral presentations were held in this topic at the 24th Congress of the International Society of Thrombosis and Hemostasis, 2013, Amsterdam, The Netherlands and at the 23th Biannual International Congress on Thrombosis, 2014, Valencia, Spain and at the 12th Congress of the Hungarian Society of Thrombosis and Haemostasis, 2014, Szilvásvárad. (*Thromb Res 2014; 133:S11-12. and OC90.3, http://www.eventure-online.com/eventure/publicSession.do?id=208557&test=208557 and Clin Chem Lab Med 2014;52:eA86-7, Metabolizmus 2014;XII: 261*).

The high number of ATBp3 patients allowed us to study genotype-phenotype associations. Altogether 102 patients belonging to 63 families were registered as ATBp3 carriers. Among them 26 homozygotes and 76 heterozygotes were identified. Most of the homozygotes suffered at least one episode of venous thrombosis (VT), only two of them remained symptom free to date, however one of them is still very young (10 years of age). The median age at the first thrombotic episode was 14.5 years (2 days to 26 years). Eight patients suffered recurrent VT, and PE was confirmed in 4 patients. Three females had obstetric complications (fetal death) in their case histories. The presence of additional inherited risk factors of VT was registered only in three patients (one prothrombin

20210G>A heterozygote and two Factor V Leiden heterozygotes). Three patients were confirmed as having congenital vascular anomaly. No provoking factors were registered in our patients except for two females who suffered at least one VT episode during pregnancy. Among the registered ATBp3 heterozygotes both symptom-free individuals and patients with thrombosis are represented. Thirty-seven patients out of the 76 ATBp3 heterozygotes had at least one VT episode. The median age at the first thrombosis was 33 years, however a wide range of age was registered between 2 and 68 years. Only 5 patients had thrombosis before the age of 20 as opposed to the ATBp3 homozygotes, among homozygotes almost every patient suffered the first thrombotic event before 20 years of age. Among ATBp3 heterozygotes 6 had recurrent VT. PE occurred in 13 patients and surprisingly, arterial thrombosis was in the case history of ten individuals. Among the female patients (n=34) seven suffered from spontaneous abortions. Approximately half of all ATBp3 heterozygotes with thrombosis had at least one provoking factor or acquired risk factor. Thirty-four percent of the patients with thrombosis had other inherited risk factor; 14 patients were carriers of either Factor V Leiden (FVL) or prothrombin 20210A.

In summary, we used nine genetic markers in ATBp3 carriers and 200 healthy controls and undoubtedly confirmed the founder effect of ATBp3 mutation. Our study is the first in which the clinical phenotype and thrombosis risk conferred by ATBp3 mutation is clarified in a large population. The risk of thrombosis in homozygous individuals seems to be very high, it is significantly higher than that of heterozygous patients (OR 14.06; 95%CI 3.10-63.74). The spectrum of thrombotic symptoms was found to be wide in our patients; severe thrombosis often in unusual localization was registered in many homozygotes and pregnancy complications were also common. Based on this study and recent findings of others it seems to be useful to look for AT deficiency at least in a selected patient population with obstetric complications or arterial events. *(J Pediatr Hematol Oncol. 2012; 34: 276-9., Thromb Res. 2014;133:1158-60.* and *R. Gindele, M. Speker, Á. Udvari, A. Selmeczi, Z. Oláh, P. Ilonczai, G. Pfliegler, E. Marján, B. Kovács, Z. Boda, L. Muszbek and Z. Bereczky. Antithrombin Budapest 3 mutation caused by a founder effect in the Hungarian population; genotype-phenotype relations investigated in a large antithrombin deficient population; J. Thromb Haemost, 2015, submitted)*

I/2 and I/3 Protein C (PC) and Protein S (PS) deficiencies:

Since the two proteins act together as natural anticoagulants and the effect of Factor V Leiden mutation (FVL) on laboratory assays detecting PC and PS deficiencies is known, hereby we report our results concerning analysis of the laboratory results from the point of view of FVL and PC/PS gene mutations together. In the laboratory diagnosis of PC/PS deficiencies the first line test is a functional assay, which is based on clotting time determination (in case of PC and PS) or on amidolytic activity of PC detected by the change in optical density (chromogenic assay of PC). The presence of FVL influences the clotting assays leading to spuriously low PC or PS activity levels. The chromogenic functional assay for PC and all antigen assays are free from this interference. Therefore in FVL carriers the misdiagnosis of type II PC, or PS deficiencies is a common problem especially in populations where FVL is very frequent, like in Hungary. During the study

period individuals having low PC activity by the clotting method (Siemens, Protein C coagulometric, cut off value below 70%) were recruited. By the end of enrollment 180 individuals were registered and protein C gene (PROC) mutation, clinical and laboratory data and the presence or absence of FVLeiden were registered. Chromogenic PC activity was determined by Siemens reagent. After exclusion of patients having normal PC activity result in the repeated testing or having acquired PC deficiency n=144 (n=126 non-related probands) patients remained in the study. Thirty-seven out of the 126 non-related patients had PROC mutation detected by direct DNA sequencing. Four additional patients had larger gene segment mutations as detected by MLPA analysis. When divided the patients according to FVL positivity/negativity it was observed that the majority of patients had FVL mutation (FVL+, n=77). Among patients without carrying FVL (FVL-, n=49) the mutation detection rate was quite high, 69.4% and the causative mutation was not found only in 15 cases (Figure 5). However, comparing it to AT deficiency, where the causative mutations were practically identified in all patients with low AT levels, this should be considered as more complex situation and the reason for it is to be investigated in future studies more deeply.

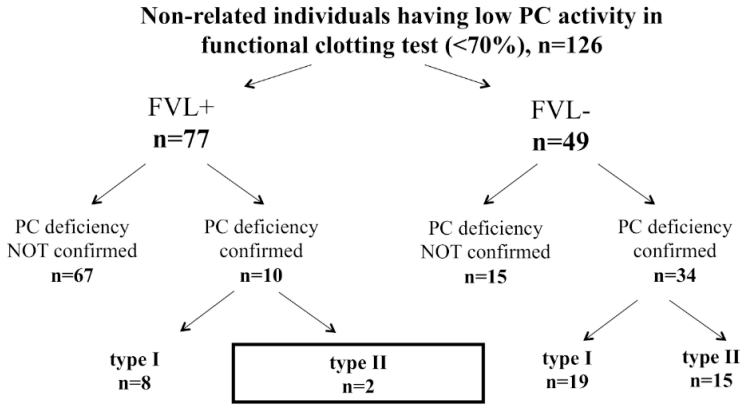

Figure 5. Genetic analysis of patients with low functional clotting PC activity.

Among FVL- patients with PROC mutations the ratio of type I and type II PC deficiencies was close to one. Among FVL+ patients the mutation detection rate was extremely low (13%), as expected; two patients had type II PC deficiency and 8 patients had type I PC deficiency. The frequency of type II PC deficiency in patients with PROC mutations was 39%. By performing PC antigen concentration, type I and type II deficiencies are well distinguished. In the presence of FVL and normals PC antigen the diagnosis of type II deficiency is problematic. In our study, among FVL+ patients practically all patients, except for the 8 type I deficients and 6 additional patients within the group without PROC mutation, had normal PC antigen concentration. It is to be noted, however, that in FVL+ group with normal PC antigen only two patients with true PC deficiency was identified. Concerning the usefulness and safety of chromogenic assay as first line test instead of clotting assay in PC deficiency we have observed that chromogenic test gave abnormal result in all type I PC deficient cases, in which case the presence or absence of FVL did not matter. In type II deficiency seven out of the 17 individuals had normal chromogenic PC activity (false negativity rate 41%). Based on these results a diagnostic protocol was suggested, in which the clotting assay for

screening PC deficiency is not omitted, but chromogenic assay is considered as second line test. Oral presentations were held in this topic at the 24th Congress of the International Society of Thrombosis and Hemostasis, 2013, Amsterdam, The Netherlands, at the 12th Congress of the Hungarian Society of Thrombosis and Haemostasis, 2014, Szilvásvárad, Hungary and at the 57th Congress of the Hungarian Society of Laboratory Medicine, (*OC78.2, http://www.eventure-online.com/eventure/publicAbstractView.do?id=216760&congressId=6839., Metabolizmus 2014;XII:247 and Clin Chem Lab Med 2014;52:eA68-9*)

Individuals having 65% or lower PS activity measured by the clotting test were recruited after excluding the presence of acquired protein S deficiency (PSD). By the end of the study n=132 non-related (altogether 166 with relatives) patients were examined according to the same scheme as in the case of PC. PS activity was measured by Siemens PS reagent, free PS antigen was measured by immunoturbidimetry (Siemens). More than 50% of patients with low PS activity were carriers of the FVLeiden (FVL+, n=73) and type I PSD was suggested in half of them (Figure 6). In contrary, among non-carriers (FVL-, n=59) the laboratory results suggested type I PSD in 83%. In FVL- 24 patients had PROS1 mutations. Among them 3 mutations were detected by MLPA suggesting larger gene deletions (delE4-6, delE1, delE7). Known polymorphisms were identified in 30 patients. In FVL+ 14 mutations were found. Two novel mutations involved deletion of larger gene segments, as detected by MLPA (delE9 and dupE12). In FVL+ individuals the ratio of causative mutations was markedly lower than in FVL- subjects (19% vs. 41%). Among patients with causative mutations 2 (FVL-) and 3 (FVL+) had type II PSD. In summary, the majority of patients with FVL+ showing type II PSD laboratory phenotype were negative for PROS1 mutation. Hovewer, the high rate of mutation negative cases in FVL- suggests that beyond MLPA, as second line genetic diagnostic tool, larger gene/chromosome alterations or epistasis should be hunted for. Concerning the alternative functional PS assays, which may be free from FVL interference one possible solution was published soon after the beginning of the research project by Tsuda et al (Blood Coag Fibrinol 2012;23:56-63) and a collaboration with them was established. Testing of our samples from the point of view of FVL interference is very promising. Presentations were held at the 23th Biannual International Congress on Thrombosis, 2014, Valencia, Spain and at the 57th Congress of the Hungarian Society of Laboratory Medicine, Hungary (*Thromb Res 2014;133:S81 and Clin Chem Lab Med 2014;52:eA68*) and manuscript describing our considerations concerning PC and PS diagnostics is under preparation.

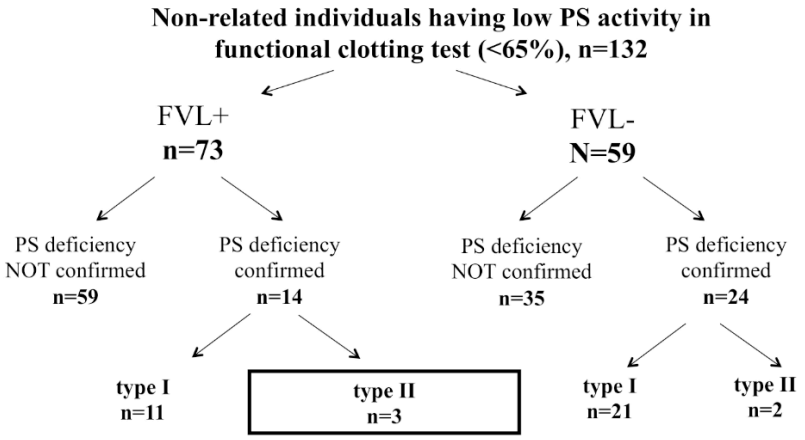

Figure 6. Genetic analysis of patients with low functional clotting PS activity.

II. Investigation of the consequences of novel mutations at molecular level

1, Molecular characterization and clinical evaluation of AT p.L173P (p.L205P according to the HGVS nomenclature) mutation.

This novel mutation affected one family with severe thrombotic symptoms and thirty family members of four lineal generations could be included in the family study. The age at the first thrombotic event, the type and location of the thrombosis, the recurrent thrombotic episodes, and the co-existing provoking factors and acquired conditions were determined in every family member. We found ten affected patients in the family and all of them suffered from venous thromboembolisms except for two teenage boys in the youngest generation. In six cases the thrombosis was recurrent. Two of the patients died of pulmonary embolism, one of them at the age of 19. The mean age at the time of the first thromboembolic episode was 36.5 years. The hc-anti-FXa AT activity and p-anti-FXa AT activity values of affected patients were decreased and AT antigen was also decreased corresponding to type I deficiency. HEK293 cells were transfected with constructs containing wild type and Pro205 SERPINC1 plasmids purchased from ImaGenes GmbH (Berlin, Germany). Transfection efficiency was determined by beta-galactosidase assay. Western blotting was used to visualize the presence or absence of the mutant AT in the cell lysates and in the conditioned media. The quantity of the expressed AT protein was measured by ELISA. Double immunofluorescent staining and confocal laser scanning microscopy (CLSM) analysis was executed for wild type and mutant AT plus endoplasmic reticulum/cis-Golgi/trans-Golgi and late endosome/26S proteasome markers. Mutant AT was detected from the cell lysates in the same amount as wild type, however less than 30% of the wild type was measured in the conditioned media suggesting impaired secretion. CLSM experiments demonstrated that mutant AT accumulated at the trans-Golgi-late endosome and as a result of deviation from the normal secretory pathway, it reached the 26S proteasome. Quantitative analysis of co-localization studies was performed by the protein proximity analyzer software as it was described by Wu Y. et al (Biophys J. 2010;98:493-504.) (Figure 7). To study the possible structural alterations caused by the mutation the mutant AT protein structure was derived

from the wild type one, deleting first the 205Leu side chain manually then rebuilding it as 205Pro residue by means of the YASARA software, then molecular simulations were carried out by means of the Gromacs 4.5.4 package. Protein structure visualizations were done by the VMD 1.9.1 or the CHIMERA software tools. The analysis of the trajectories obtained from 4μs dynamics simulations for the wild type and the 205Pro mutant protein showed that in the surrounding region of position 205 a large geometry distortion took place. This was especially observed at the N- and the C-terminal end of the helix F, which lost its helical character. These results support the conclusions drawn from biochemical studies, that the mutant AT –due to structural abnormality- suffers secretion defect, intracellular accumulation and may be degraded by intracellular proteases. Oral presentation was held in this topic at the 24th Congress of the International Society of Thrombosis and Hemostasis, 2013, Amsterdam, The Netherlands and at the 12th Congress of the Hungarian Society of Thrombosis and Haemostasis, 2014, Szilvásvárad, Hungary, manuscript has been finished for submission to Thrombosis and Haemostasis. (*OC78.3, http://www.eventure-online.com/eventure/publicAbstractView.do?id=217429&congressId=6839 and Metabolizmus 2014;XII:249*).

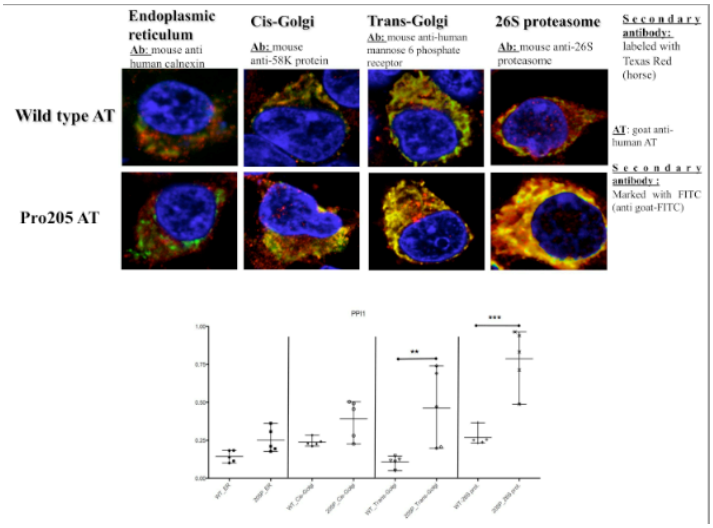

Figure 7. Intracellular localization of wild type and 205Leu AT.
By performing double immunofluorescent staining and CLSM analysis mutant AT showed co-localization with the trans-Golgi and the 26S proteasome, which is indicated by the yellow color in the merged pictures (above). By means of protein proximity analyzer software co-localization was quantified and expressed as "protein proximity index, PPI", which refers to the "fraction" of AT co-localizing with the certain cell organelles. In the case of trans-Golgi and 26S proteasome PPI of 205Leu AT was significantly higher than that of wild type (trans-Golgi: median 0.5 (range 0.2-0.7) and 0.1 (range 0.05-0.14) for mutant and wild type, respectively and 26S proteasome: median 0.9 (range 0.7-0.98) and 0.26 (range 0.23-0.36) for mutant and wild type AT, respectively).

2, Molecular characterization and clinical evaluation of AT p.Asn418Ile and p.Gly424delinsAla-Thr mutations.

Instead of characterizing PS p.Pro42Thr mutation (as it was planned originally) we have performed the molecular investigation of two additional novel AT mutations which were found during the project because didactically it seemed to be better choice from the point of view of publication. The p.Asn418Ile and p.Gly424delinsAla-Thr (p.Asn450Ile and p.Gly456delinsAla_Thr according to HGVS nomenclature) were detected in one family each. The p.Asn450Ile mutation was detected in four family members; three of them has already suffered VTE and AT activity values were around 50% with proportionally low AT antigen levels suggesting the presence of heterozygous type I deficiency. The p.Gly456delinsAla_Thr mutation was detected in only one individual with VTE, her AT activity was 54%, AT antigen was 0.15 g/L, which also suggested type I deficiency. Following the above-mentioned research protocol, after site-directed mutagenesis and transfection into HEK cells the Ile450 AT was demonstrated in a very small amount both intra-and extracellularly (10 and 8% of wild type AT, respectively). The 456Ala-Thr was present in the cells in 30% of the wild type, however it was undetectable in the media. In double immunofluorescent studies, as it was expected due to the very low amount of mutant AT molecules, low level of co-localization was observed in case of every cell organelles. The mRNA quantification studies were carried out on LightCycler 480 in duplicates using SYBR Green I Master (Roche). Gene expressions were normalized to the expression level of Ornithine decarboxylase antizyme 1 (OAZ1). PCR reactions were set up at final volume of 20 µl and consisted of 10 µL Master Mix (2x oncentration), 5 µl of cDNA template derived from reverse-transcribed RNA, 300 nM primers for the reference gene (OAZ1) and SERPINC1. In the case of p.Gly456delinsAla the normalized quantification cycle (Cq) value (that corresponds to the number of cycles at which the fluorescence signal exceeds a threshold value) was significantly higher than that of wild type AT, which suggested defective mRNA synthesis from the mutant SERPINC1 gene. In the case of p.Asn450Ile mutation the Cq value did not differ significantly from the normal suggesting normal mRNA synthesis and alteration at a later stage of protein synthesis (*Clin Chem Lab Med 2014;52:eA66*).

3, Molecular characterization and clinical evaluation of p. Asp35Gly, p. Ala121Val and p.Ala121Glu (p.Asp77Gly, p.Ala163Val and p.Ala163Glu according to HGVS nomenclature) mutations causing protein C deficiency.

In this study a newborn with purpura fulminans and a patient with deep vein thrombosis at the age of 27 were investigated. The first patient had PC activity below 1% and PC antigen concentration was 5% and she was a compound heterozygote possessing the p.Asp77Gly mutation and the p.Ala163Glu mutation. PC activity was 49 % and the PC antigen concentration was 50% in the case of the second patient and she was heterozygous for the p.Ala163Val mutation. After invitro expressing the wild type and mutant constructs in HEK293 cells the antigen concentration of the wild type and mutant PC in the cell lysates did not show marked difference. In the media, the concentration of the 77Gly mutant was measurable, however it was somewhat lower than the concentration of wild type protein, while the 163Val and 163Glu mutants were undetectable. The same results were obtained by Western blotting. PC activity of 77Gly in the medium by performing the amidolytic assay was comparable with that of wt PC in

three separate experiments (77.5%±15.1%, when PC activity of the wt PC was considered as 100%). Moreover the specific activity (ie. the activity related to one mg PC protein) of 77Gly was the same as wt PC (104.2%±28.4%, considered wt PC as 100%). No activity could be detected in the media of the Mock, the 163Val and the 163Glu mutants. By performing the clotting assay, the PC activity of 77Gly was 80%±9.4% of wt PC. Again, no activity was measured in the mock, the 163Val and 163Glu samples. To detect if there is any difference in the speed of activation between wt and 77Gly PC, medium samples have been pre-incubated with Protac for various times from 1 to 10 minutes and clotting times have been measured by adding APTT reagent and calcium-chloride to the samples. No difference was shown between wt and 77Gly mutant PC in this aspect. The intracellular localization of the wt, 163Val, 163Glu and 77Gly mutant proteins was visualized by immunfluorescent staining followed by CLSM and quantified by the calculation of colocalization coefficients. 77Gly PC did not show marked colocalization with either cell organelles; 163Glu and 163Val mutants showed accumulation in the 26S proteasome. For detecting the ratio of polyubiquitinated PC in the cell lysates of transfected cells an ELISA method was established in which PC of the cell lysates were captured by polyclonal anti-PC antibody (Diagnostica Stago) coated to the microtiter plate and polyubiquitin was catched by a horseradish peroxidase conjugated anti-polyubiquitin monoclonal antibody (CycLex Co. Ltd., Ina, Nagano, Japan). The reaction was developed by adding tetra-methylbenzidine (TMB) substrate and the optical density was red at 450 nm in a microplate reader. Poly-ubiquitination of mutant proteins was calculated after normalizing the OD values for PC antigen concentration in each cell lysates and expressed as a ratio of mutant to wt polyubiqutinated PC. The ratio of polyubiquitinated mutant PC to wt was more than two in the case of 163Val and 163Glu mutants (2.25±0.49 and 2.95±0.51 for 163Val and 163Glu, respectively), while the 77Gly mutant was not polyubiquitinated (ratio of 77Gly to wt was 0.96±0.10). By performing molecular modeling studies and simulations it was demonstrated that the positions of the mutant 163Glu and 163Val residues changed significantly during the simulation period as opposed to the wild type 163Ala suggesting the possibility of altered folding. A possible consequence of this wrong folding can be the prevention of the disulfide bridge formation. In the case of 77Gly no major structural abnormality was revealed. As a conclusion of our results summarized above, 163Val and 163Glu PC was detected with the marker of the 26S proteasome, which serves as a potential site for intracellular degradation of misfolded proteins after polyubiquitination. These results were supported by demonstrating the higher polyubiquitination level of 163Val and 163Glu, which also serves as an indirect evidence of intracellular degradation. In summary, our results indicated that p.Ala163Glu and p.Ala163Val mutations lead to structural abnormality and secretion defect of PC. In the case of 77Gly PC no severe secretion defect was confirmed. Moreover both the amidolytic and clotting functional PC assays showed normal activity of the secreted 77Gly protein. It was also demonstrated that the rate of activation of 77Gly by Protac did not differ from the wt PC. Double immunofluorescent staining showed no colocalization with either cell organelles and no increased polyubiquitination was detected. Putting all our findings together, p.Asp77Gly mutation leads to a PC with normal FVa and FVIIIa inactivation effect, and as no marked secretion defect could be seen, it might have an impact on the intermolecular interactions in which PC is involved and also on the clearance of PC.

These hypotheses could be an interesting subject of further research. We have published these results most recently (*Thromb Res 2015; 10.1016/j.thromres.2015.01.011*).

4, Molecule modeling studies and dynamics simulations in AT.

Two μs molecular dynamics simulation were carried out for the native free forms of antithrombin (AT). Constant particle number (N) constant pressure ($P=10^5$Pa) and constant temperature (T=310K) molecular dynamics simulations with periodic boundary (PBC) condition were carried out by means of the GROMACS software package using the AMBER ’03 force field. For the water the TIP3P explicit water model were applied. For the short range electrostatic and van der Waals energy terms 10Å cut-off distances were used. The long-range electrostatic energy corrections were calculated by means of the particle mesh Ewald (PME) method. The v-rescale and Berendsen algorithms were used for temperature and pressure couplings, respectively. In order to apply a longer time step (4fs) during simulations virtual sites protocol was used. By the evaluation of simulation trajectories it was pointed out, that the reactive center loop (RCL) can populate conformational states with solvent exposed Arg393 side chain conformation, which can form the AT-protease Michaelis complex. It was shown also that the peptide sequences corresponding to the helix D extension can be featured by especially large root mean square fluctuations populating even helix-like conformational states. In the simulation where the helix P still not formed, the corresponding sequence showed especially large fluctuation as well. Mutual information analyses showed remarkable (generalized) correlation between those regions, which found to change their conformation and/or orientation in X-ray structures of AT-PS complexes. It has been revealed that allosteric information propagation pathways exist even in the non-activated native form of AT. The same conclusion can be drawn by principal component analysis extracting the principal components of fluctuations from the trajectory of simulation as well as weighted implementation of suboptimal path (WISP) analysis of dynamics trajectories. These results serve as good basis for future research concerning functional studies in AT deficiency and has been published recently (*J Biomol Struct Dyn;2015, DOI:10.1080/07391102.2014.986525*).

Finally, based on our experience on the inhibition of AT on FXa we have participated in a study concerning the effect of modified pentasaccharides on FXa inhibition. In this study two pentasaccharide sulfonic acids were prepared with modifications in primary sulfate esters (two, or three primary sulfate esters were replaced by sodium-sulfonatomethyl moieties). In vitro evaluation of their anti-FXa activity in the presence of AT was tested and it was found that the disulfonate analogue exerted a more efficient anti-FXa effect, while the trisulfonate analogue seemed to be a weaker inhibitor than the original idraparinux molecule. It was suggested that this difference could be because of the different conformation of the L-iduronic acid residues of these bi-and trisulfonic acid counterparts of these analogues (Chemistry 2012;18 (34):10643-52).

III. Investigation of AT, PC and PS polymorphisms:

Enrollment of VTE patients and healthy controls has been finished in the last year. Clinical and laboratory data as described in the work plan were collected and registered. Using self-designed primers and various size genotyping oligonucleotides multiplex PCR

followed by primer extension assay and fluorophore-labeled ddNTP for detection by fragment analysis on a 3130 Genetic Analyzer was established. This new assay is able to detect 12 SNP's simultaneously, as follows, PROC rs1799809 (IVS1-1641A>G), rs1799808 (IVS1-1654C>T), rs1799810 (IVS1-1476A>T), rs2069928 (IVS7+111G>T) and rs1401296 (3'UTR C>T); PROCR rs867186 (p.Ser219Gly), rs6088735 (5'UTR C>T) and rs8119351 (5'UTR G>A), SERPINC1 rs2227589 (IVS1+141G>A) and rs121909548 (p.Ala384Ser), PROS1 rs8178649 (IVS11+54T>C) and rs121918472 (p.Ser501Pro). Briefly, amplification of the 12 corresponding PCR fragments were executed in 4 separate multiplex PCR, then in the primer extension assay unlabeled genotyping primers with different length polyGACT tail are added to the PCR products to anneal to the sequence adjacent to the corresponding SNP sites (Figure 8).

Primer mix		Gene	Localisation	Genotype	PCR product(bp)	Genotyping primer (bp)	Sequence of genotyping primer	Allele frequency in European population
M1	7	PROCR	5'	C>T	185	50	$(GACT)_6$CTGCTTTCTCAAGTATGTTATATTCT	73%>21%
	9	SERPINC1	IVS1+141	G>A	240	59	$(GACT)_9$AGTCAAAGACCTGTTTGGAAGAC	90%>10%
	12	PROS1	Ser501Pro Heerlen	T>C	280	71	$(GACT)_{12}$TACATGCCAACCCTCAGCACTGG	99,6%>0,3%
M2	2	PROC	IVS1-1654	C>T	307	28	TTTTGCCCTCACCTCCCTCCCTGCTGGA	68%>32%
	4	PROC	IVS7+111	A>G	202	34	$(GACT)_4$CCCACCTTCATCATCCCCCAAA	84%>16%
	6	PROCR	Ser219Gly	C>T	250	44	$(GACT)_6$CACACCAGCAATGATGAAAC	91%>9%
	8	PROCR	5'	G>A	168	55	$(GACT)_8$CAAAGGGAAATGAAGGAAGCAAG	90%>10%
M3	3	PROC	IVS1-1476	A>T	140	23	GGGTCGTGGAGATACTGCAAGT	65%>35%
	5	PROC	3'	T>C	278	38	$(GACT)_5$GCCCTGTGCCATTCGGC	61%>39%
	10	SERPINC1	Ala384Ser Cambridge	G>T	221	63	$(GACT)_{11}$ATCACAACAGCGGTACTTG	99,9%>0,1%
M4	1	PROC	IVS1-1641	A>G	236	18	CTGGACGGCATCCTTGGT	52%>48%
	11	PROS1	IVS11+54	T>C	173	67	$(GACT)_{11}$GGATGAGTTCCTTTTGTCTGTAA	78%>22%

Figure 8. Characteristics of the 12 detected SNP's and the genotyping oligonucleotide primers.
The genotyping primers have a so called poly(GACT) tail at the 5' end, which allows to separate the 12 different SNP's by capillary electrophoresis. The numbers in subscript after "GACT" correspond to the respective repeat number.

After annealing of the genotyping primers to the PCR products a secondary PCR, called primer extension step occurs as a single-base extension by adding fluorescently labeled ddNTP (dye-terminator). As each base (A, T, C and G) is labeled by different fluorescent dyes, after adding the base complementary to the site of the SNP to the genotyping primer will yield marker fragments for the different SNP alleles that are all the same length but vary by color. After capillary electrophoresis and fluorescent detection, the alleles of a single marker appear as different colored peaks at the same size in the electropherogram. For the detection reaction the SNaPshot Multiplex kit (Life Technologies) was used. The newly developed method was verified by DNA sequencing in DNA samples of 20 healthy volunteers, then all VTE patients and control subjects were genotyped. Median age of VTE patients (n= 304) was 57 years (range 19-88 years), while it was 31 years (range 18-69) in the case of healthy controls (n=278). Among

patients the median age at the first thrombotic event was 38 years (interquartile range 28-53). Twenty-nine precent of the patients had recurrent VTE, among them 12 patients had more than 2 thrombosis. Twenty-five percent of the patients suffered from PE and 35% had positive family history. Only 7 patients were identified with known causative mutations within SERPINC1 and PROC (5 patients carried the ATBp3 in heterozygous form, 2 patients had heterozygous pathogenic mutations in PROC). FVL was carried by 18.3%, while prothrombin 20210A was carried by 2.7% of the patients. Eighteen percent of the patients had elevated fibrinogen and 30% was registered with elevated FVIII. Concerning the investigated polymorphisms the PS Heerlen polymorphism (p.Ser501Pro) was carried only by 2 individuals in the patients' group. AT Cambridge (p.Ala384Ser) was absent in our study population. Since this latter polymorphism is a common variant in some western populations we have established a real-time PCR and melting curve analysis method for detecting this variant in 1000 healthy persons recruited by the Hungarian General Practitioners' Morbidity Sentinel Stations Program (HMSSP). This variant was also absent in this population. The minor allele frequency (MAF) values in the case of all other SNP's were similar to the MAF values for Caucasians as reported in the HapMap database. Strong, however not 100% linkage was observed in the case of PROC and PROCR polymorphisms. Carriers of PROCR p.Ser219Gly and PROC rs1401296 were in average 7 and 5 years younger at the time of first VTE than non-carriers. Moreover, after age stratification it was demonstrated that below the age of 50 years rs1401296 was significantly more common in patients having VTE (p=0.003) and conferred an almost two-fold risk of VTE (OR 1.98, 95%CI 1.27-3.10). In the case of FVL carriers (n=94) PROC rs2069928 increased the risk of recurrent thrombosis by 2.98-fold (95%CI: 1.11-7.50). In our study population SERPINC1 and PROS1 polymorphisms were without any effect on VT or PE development and recurrent thrombosis. In summary, in our population some polymorphisms within PROC and PROCR genes seem to influence VTE development and/or the development of recurrent thrombosis. These results reflect to the potential role of common variations in these genes in thrombotic diseases and suggest the need for further research to clarify the biochemical background of these phenomena. Oral presentation was held in this topic at the 12th Congress of the Hungarian Society of Thrombosis and Haemostasis, 2014, Szilvásvárad, Hungary (*Metabolizmus 2014;XII:253-4*), the manuscript is under preparation.

In conclusion this OTKA project added important new pieces of information to the field of thrombosis research and laboratory diagnostics and provided a good basis of future research and an OTKA K proposal.