

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**GENOTYPE-PHENOTYPE CORRELATIONS IN
HEMOSTASIS DISEASES; ANTITHROMBIN DEFICIENCY
AND OSLER-RENDU-WEBER DISEASE**

by Réka Gindele

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF KÁLMÁN LAKI

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Head of the **Examination Committee**: Prof. Pál Soltész, MD, PhD, DSc

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The Examination takes place at Bldg. C, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 11:00 am, 26th of March, 2018.

Head of the **Defense Committee**: Prof. Pál Soltész, MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:00 pm, 26th of March, 2018.

1. INTRODUCTION

The proper functioning of the coagulation system requires a coordinated balance between the 3 pillars of the hemostasis system: the vascular wall (vascular system), the blood clotting components (humoral system) and platelets (platelet count and quality, as cellular system).

Various genetic variants of the procoagulant, anticoagulant and fibrinolytic factors involved in blood coagulation may predispose to either bleeding or thrombosis. In case of the AT deficiencies, well-characterized subtypes exist according to the localization of the causative mutation in AT gene (*SERPINC1*). These AT deficiencies can be classified into quantitative (type I) and qualitative (type II) subgroups. In type I, as a result of defective protein synthesis or secretion, AT activity and antigen levels are both decreased. In type II deficiency, the ineffective protein decreases AT activity and leads to normal or slightly reduced antigen levels. The 3 subtypes of type II AT deficiency may affect the heparin binding site (II.HBS), the reactive center (II.RS) or could be a pleiotropic (II.PE) defect. Up to 310 different mutations have been identified in the *SERPINC1* gene so far. The different AT deficiency subtypes do not necessarily have the same clinical phenotype, so far the data available in the literature is sparse; partly case reports, partly small population studies have been reported. This might be due to the rare occurrence of AT deficiency (1: 2000 - 1: 5000), but there is not enough evidence for confirmation. In addition, even within the same subtype, there may be phenotypic differences that are characteristic of each mutation. Laboratory diagnostics of AT deficiency are based on a functional test in which the inhibition of AT on active factor X (FXa) or thrombin (FIIa) is investigated in the presence of heparin. It has been observed that the currently available functional tests in certain AT deficiency subtypes have different sensitivity, which makes the laboratory diagnosis extremely difficult. Evaluation of each test is therefore an important task and there is currently insufficient data in this aspect.

The occurrence of frequent mutations within a population can be the result of a founder effect. In the case of AT deficiency, three mutations have been diagnosed and verified as founder mutations. Identifying founder mutations in a population is useful both in diagnostic and therapeutic procedures. In the case of new mutations not yet reported in the literature it is important to demonstrate the causative nature by both indirect and direct (biochemical) methods; also the characterization of these mutations can provide interesting data about the structure, function and interactions of the respective protein.

The vascular wall plays a central role in the coordination of hemostatic responses in the event of vascular damage. The vessels are dynamic units involved in a variety of different activities including vasomotor function, maintaining proper blood pressure, selective permeability of cells and solutes between blood and surrounding tissues, congenital and

acquired immunity, regeneration and repair, and hemostasis regulation. Vascular abnormalities can be grouped in a number of different ways, such as etiology, for inherited or acquired forms. The most prominent hereditary vascular diseases are connective tissue disorders, arteriovenous malformations (AVM), hereditary hemorrhagic telangiectasia (TA), and cerebral cavernous abnormalities. Hereditary hemorrhagic telangiectasia (HHT, Osler-Rendu-Weber disease) is a multisystemic vascular abnormality; the most common symptoms are spontaneous recurrent nosebleeds, TAs occurring in the skin and mucous membranes and AVMs with bleedings in the lung, brain and gastrointestinal tract (GI). Mutations of three genes were significantly associated with Osler's disease. The most commonly involved is the *ENG* gene encoding the endoglin protein, a major glycoprotein of the endothelium. The other often affected gene is the Activin receptor-like kinase 1 (*ACVRL1*) gene expressed in endothelial cells. The third most common HHT related gene is *SMAD4* (Mothers against decapentaplegic homologue 4). The *SMAD4*-encoded protein is an intracellular signal molecule on the transforming growth factor beta (TGF β) and the bone morphogenetic protein (BMP) pathway. The Osler-Rendu-Weber disease is an autosomal dominant disorder; the mutation spectrum differs in different populations, and hot spots are not identified in these genes.

In this thesis, the results of the genetic, clinical, laboratory and protein biochemical analysis of AT deficiencies and the results of our genetic analysis of HHT are summarized.

2. REVIEW OF LITERATURE

2.1 The role of antithrombin in coagulation

2.1.1 Structure, function and genetic characteristics of antithrombin

Antithrombin (AT) is a single-chain glycoprotein belonging to the serpin family, synthesized in the liver, the molecular weight of it is 58,200 Da. After the cleavage of the propeptide (32 amino acids), the mature protein is composed of 432 amino acids. Two glycoforms of AT are present in the circulation, the majority as α -glycoform (90-95%). β -glycoform is present to a lesser extent (<10%).

The human AT gene (*SERPINC1*) is located at 1q23-q25, containing 7 exons and 6 introns resulting in 1.4 kb mRNA. Exons 2 and 3 encode the heparin binding region, while the catalytic core is encoded in the C' terminal of exon 7. Nine complete and one partial *Alu* repeat sequences were identified in the 1, 2, 4, 5, and 6 introns. Several single nucleotide polymorphisms (SNPs) are known throughout the gene, which are generally harmless and occur at different frequencies in various populations.

AT is a serine protease inhibitor that inhibits both thrombin and the enzymes responsible for thrombin generation. It is a crucial endogenous anticoagulant molecule, which explains, why even mild AT deficient patients (heterozygotes) have a high thrombotic risk. The heparin-induced anticoagulant activity comes from its ability to enhance the anticoagulant effect of the AT protein. AT is a progressive inhibitor because its ability to react with the active coagulation factors is small, but the inhibitory effect increases to 500-fold in the presence of heparin or heparan sulfate proteoglycan (HPSG). In addition to its well-known anticoagulant activity, AT also has anti-inflammatory, antiproliferative, antiangiogenic and antiviral properties.

AT has an important regulatory role in hemostasis and thrombosis, by inhibiting [a] thrombin-mediated fibrin clot formation, [b] active factor X (FXa) mediated thrombin generation and [c] clotting factors that are higher up in the intrinsic and extrinsic pathways (FIXa, FXIa, FXIIa, plasma kallikrein, and FVIIa tissue factor (TF) complex).

2.1.2 Molecular genetic background of antithrombin deficiency, genotype-phenotype correlations

The first case of AT deficiency was described by Egeberg et al. in 1965 and the first functional AT defect, named as AT Budapest, was reported by Sas et al. in 1974. AT deficiency is classified into type I (quantitative) and type II (qualitative). In type II deficiency, the defect may involve the reactive site (II.RS), the heparin-binding site (II.HBS) or it can exert a pleiotropic effect (II.PE).

The inheritance of AT deficiency, in general, is autosomal dominant. The majority of AT deficient patients are heterozygous for the defect with typical AT activity values approximately 50%. Homozygosity is incompatible with life, with the exception of type II.HBS variant. The molecular genetic background of AT deficiency is heterogeneous; more than 310 mutations were described in the database of human gene mutation data (HGMD) (<http://www.hgmd.cf.ac.uk>). The most common variations, such as AT Basel, (p.Pro73Leu), AT Padua I (p.Arg79His) and AT Budapest 3 (AT Bp3, p.Leu131Phe) mutations, lead to type II.HBS deficiency.

Most of the genetic defects result in highly increased thrombotic risk at a young age in type I deficiency and in most type II deficiencies, with notable exception of type II HBS variants. The p.Ala416Ser (AT Cambridge II) is an exception to the II.RS deficiencies, as it may also occur in homozygous form and associated with a mild clinical phenotype. The II.HBS subtype is likely to have a lower thrombotic risk than other subtypes. Individuals with homozygous II.HBS mutation are alive, however thrombosis develops at an early age, while heterozygous patients suffer thrombotic events at older age.

2.1.3 Consequences, epidemiology and therapy of antithrombin deficiency

Thrombotic episodes are mainly confined to the venous system in individuals with genetic AT deficiencies. Common problems include recurrent episodes of deep vein thrombosis (DVT), mesenteric thrombosis and/or pulmonary embolism. DVT can often occur in unusual localization, such as upper limbs, mesenteric, kidney, portal, retinal and cerebral vessels. In some cases, arterial thrombosis has also been reported. AT deficient pregnant women have an increased risk of thrombosis. One study found that AT deficient children had a high chance of ischemic stroke and cerebral venous sinus thrombosis.

Acquired AT deficiency can be observed in liver disease, nephrotic syndrome, and in renal function or protein loss disorders. Low AT concentrations can be observed in sepsis, disseminated intravascular coagulation, thrombotic microangiopathies, acute hemolytic transfusion and malignancies.

The prevalence of inherited AT deficiency in the general population is rare, it is approximately 1:2000 to 1:5000. AT deficiency is much more frequent in case of families with venous thromboembolism (VTE), it is estimated to be between 1:20 and 1:200. In several prospective and case control studies, the VTE risk caused by AT deficiency was found to be very high. Among the known thrombophilia factors, AT deficiency seems to be the most severe. AT deficiency is also a major risk factor for pulmonary embolism and recurrent venous thrombotic events (VTE).

Patients with AT deficiency who underwent VTE are usually acutely treated with heparin and heparin-AT concentrate therapy. For prophylactic anticoagulation, vitamin K antagonists or new oral anticoagulants (NOAC) are indicated. The recommended duration of anticoagulation after VTE is unclear; it is particularly difficult to choose prophylactic strategies in AT deficiency. We think that the subtype of deficiency (and possibly the identification of a specific mutation) can help solve this question; with other clinical factors taken into consideration as well. Prophylactic heparin therapy and possibly AT concentrate may be needed in asymptomatic *SERPINC1* mutation carriers in the case of pregnancy, surgery, trauma and infections. Estrogen-containing contraceptive and postmenopausal hormone replacement therapy should be avoided in AT deficient women. (Progesterone-containing contraceptives do not reduce AT levels.)

2.1.4 Laboratory diagnosis of antithrombin deficiency

In the laboratory diagnosis of AT deficiency, the first-line test is a chromogenic functional assay, in which the inhibition of thrombin (FIIa) or FXa in the presence of heparin (heparin cofactor activity, hc-anti-FIIa or hc-anti-FXa) is measured. Measurement of AT activity in the absence of heparin (progressive AT activity, p-anti-FXa) is useful for discriminating between II.HBS and other types of AT deficiency. AT antigen concentration determination also helps in classifying the AT deficiencies. Nowadays, immunonephelometry is the most commonly used method for determining AT concentrations.

Previously, human thrombin was used in the thrombin inhibitory assays, but thrombin also reacted with the heparin cofactor II, making the assay not sufficiently sensitive to AT deficiencies. Most of the commercially available assays already use bovine thrombin or FXa because they do not react with heparin cofactor II and, in principle, have similar sensitivity. In individuals with normal AT activity, currently used thrombin or FXa-based functional tests give the same results as evidenced by international quality control studies. However, in the case of AT deficient patients, depending on the type of deficiency there may be differences between in the sensitivity of the two tests. It was suggested that in the case of the AT Cambridge II (p.Ala416Ser) mutation, which is relatively common, primarily in the UK AT deficient population, the FXa based assays is not sufficiently sensitive. In the II.HBS AT deficiencies (e.g. AT Budapest 3; p.Leu131Phe), it was shown that thrombin-based tests were not, but FXa-based tests were sensitive. Lately it emerged that the sensitivity of the commercially available FXa-based tests may differ as well, but this has not been studied systematically, so far.

2.2 Hereditary hemorrhagic telangiectasia

2.2.1 Clinical features of hereditary hemorrhagic telangiectasia

Hereditary hemorrhagic telangiectasia (HHT) is a multisystemic vascular abnormality of genetic origin, characterized by aberrant direct arteriovenous connections. Telangiectases are smaller mucocutaneous lesions that appear progressively with age at characteristic sites (lips, face, hands, nasal and oral cavities and the upper gastrointestinal tract), resulting in epistaxis and, in a subset of patients, gastrointestinal bleeding. Arteriovenous malformations (AVMs) are larger, often congenital visceral lesions, located predominantly in the lungs, liver and brain. The most common symptom of HHT is spontaneous, recurrent nose bleeding. Although HHT is usually a progressive disorder, newborns are more seriously affected by lung or cerebral AVMs. The age of HHT patients is very heterogeneous at the time of diagnosis, and quite elderly patients are also present.

2.2.2 Clinical diagnosis of hereditary hemorrhagic telangiectasia

The clinical diagnosis is based on the four Curacao criteria, namely spontaneous and recurrent nosebleeds, multiple telangiectases at characteristic sites, visceral lesions and family history with a first-degree relative with HHT. Diagnosis is definite if 3 or 4 criteria are present, possible if 2 criteria are present and unlikely if fewer than 2 criteria are present.

2.2.3 Pathogenesis of hereditary hemorrhagic telangiectasia

The inheritance of HHT is autosomal dominant, with age-related and eventually near full penetrance regarding epistaxis and telangiectases. All causative genes identified to date (*ENG*, *ACVRL1* and *SMAD4*) encode proteins of the transforming growth factor-beta (TGF- β) signaling superfamily. This pathway controls the balance between the progression and resolution of angiogenesis. Approximately 85 % of HHT cases have heterozygous mutations in either the *ENG* or *ACVRL1* genes. *ENG* (localized on 9q33-q34.1) encodes endoglin, an accessory TGF- β receptor, while *ACVRL1* (localized on 12q11-q14) encodes activin receptor-like kinase 1 (ALK1), a type 1 TGF- β receptor.

No mutation hot spots were found during the analysis of these genes (HHT Mutation Database: <http://arup.utah.edu/database/hht/>). Mutations resulting in HHT are observed throughout the genes. The mutation detection rate is about 75%.

2.2.4 Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia

Pulmonary AVMs are more common among individuals carrying pathogenic *ENG* variants, whereas liver AVMs may be associated with *ACVRL1* mutations. However, both types

manifest with vascular dysplasia. Pathogenic variants of the *SMAD4* gene occur in the HHT patients mostly in combination with juvenile polyposis syndrome. Based on present data, there is no clear genotype-phenotype correlation between the clinical phenotype and the individual pathogenic variants.

2.3 Founder effect

2.3.1 The significance, consequences and methods of investigation of the founder effect

The founder effect is a special case of a change in the genetic composition of the population, it is a genetic drift. In the case of the founder effect, genetic diversity is lost, which occurs when a new population is created by a few individuals of a larger population, so the new population carries only a fraction of the genetic variants of the parental population. In isolated populations, rare alleles occur more frequently. In many isolated populations, the founding effect explains the frequent occurrences of Mendelian hereditary diseases. Numerous studies deal with common diseases occurring in Ashkenazi Jews, resulting from a founder effect, as well as the founder effect of type I tyrosinemia in Quebec's French Canadians.

In the case of diagnosis and therapy it is very useful to detect founder mutations as these enable the development of diagnostic algorithms, which can be used to simplify the diagnosis of patients in a certain population. The clinical phenotype of patients with the same founder mutation is more likely to be very similar, which makes it easier to select therapeutic or prevention strategies in the population with the founder mutation.

In the presence of common mutations, the founder effect is investigated by haplotype analysis. Because of this, SNPs and microsatellite markers are analyzed within and around the respective gene. Microsatellites or short tandem repeat sequences (STRs) are polymorphisms of simple repeating sequences occurring in different length variations. There may be mono-, di-, tri-, tetra-, penta- or hexanucleotide-length regions characterized by different alleles. After the determination of the STRs, the correlation analysis can lead to the conclusion of the age and the origin of the mutation; also genealogical analysis may occur as well.

2.3.2 Founder mutations in antithrombin deficient populations

Perry DJ et al. performed haplotype analysis and confirmed the founder effect in 18 apparently unrelated patient's DNA carrying the AT Cambridge II (p.Ala416Ser) variant. Two (ATT)_n trinucleotide STRs and 4 polymorphisms were investigated within the *SERPINC1* gene by Olds et al. to perform haplotype mapping in the Caucasian population. The haplotypes associated with mutant AT were compared in 5 family carrying AT Budapest 3 (p.Leu131Phe) and 5 family carrying p.Arg161* *SERPINC1* mutations and highlighted the

possibility of a founder effect. Similar linkage analysis was performed by Ni et al. for AT Hamilton (p.Ala414Thr) and AT Amiens (p.Arg79Cys) mutations. The AT Basel (p.Pro73Leu) is probably a founder mutation in the Finnish AT deficient population, but no genetic analysis has been performed. Extensive genetic testing for founder mutations, involving a large number of patients has not been carried out in an AT deficient population, so far.

2.3.3 Founder mutations in populations with hereditary hemorrhagic telangiectasia

HHT is widely found in all ethnic groups and continents. HHT mutations show a family accumulation; however, several, apparently unrelated patients with the same HHT mutation seem to be related to each other. If the same haplotype occurs in a non-related group of patients carrying a specific mutation, the question of the founder effect raises. In some populations, such as the people in Netherlands Antilles with two *ENG* mutations; in French and Italian HHT patients with *ACVRL1* mutations, in Danish population with *ENG* and *ACVRL1* mutations respectively, and Norwegian HHT patients with *ACVRL1* mutations, founder effects were detected.

3. OBJECTIVES

- I. Antithrombin deficient index patients and their family members were involved in the study to develop a clinical, laboratory and genetic database, in order to analyze the clinical and laboratory features of our large AT deficient population. Our goal was to find the genetic background of this AT deficient population and to describe new and common mutations. We aimed to examine the sensitivity of the functional AT assays so we could provide new data for the effective laboratory diagnosis.
- II. In our AT deficient population the frequency of the antithrombin Budapest 3 mutation was about 75%. This common occurrence raised the possibility of a founder effect, which was investigated with polymorphic genetic markers.
- III. We aimed to determine the causative nature of a new *SERPINC1* mutation by direct biochemical methods in an *in vitro* expressed mutant AT protein.
- IV. Our aim was to investigate the founder effect of the common splicing *ACVRL1* mutation in our patients with Osler-Rendu-Weber disease.

4. PATIENTS AND METHODS

4.1 Patients

4.1.1 Antithrombin deficiency

Patients with thromboembolic history and/or pregnancy complications (please see later in details) and reduced hc-anti-FXa AT activity determined from at least two independent blood samples were selected for our study. Thrombosis was described according to guidance of the International Society of Thrombosis and Haemostasis. Between January 2007 and August 2016, a total of 156 non-related AT deficient patients (index patients) and their family members (total n=246) were diagnosed at our center. All enrolled individuals were informed about the study according to the study protocol, and gave written informed consent. Ethical approval for the study was obtained from the National Ethical Council (3166/2012/HER). Patient clinical, laboratory and genetic data were recorded in a database for further evaluation.

4.1.2 Osler-Rendu-Weber disease

Patients with nosebleeds and telangiectases, characteristic of HHT (as described in the Curacao criteria) were offered to undergo a thorough general and ENT physical examination (to describe telangiectases at specific sites) and a visceral AVM screening protocol, regardless of AVM specific medical history and symptoms. This protocol includes a cranial **MRI** with and without contrast enhancement, a multi-detector chest computed tomography (CT) without contrast material and a successive contrast enhanced CT of the liver. Endoscopic evaluation of the upper digestive tract was performed when gastrointestinal bleeding was suspected or the patient had a chronic anaemia disproportionate to epistaxis.

Family history included identification of all family members with nosebleeds, telangiectases, or AVM specific symptoms. In the case of the five unrelated, HHT suspected patients a genetic test was performed for the analysis of the *ENG* and *ACVRL1* genes. Afterwards HHT-suspicious family members were also examined (n=34 individuals).

4.2 Controls

A large number of individuals (n=1000, median age 48, range 20-69 years) representing the general Hungarian population were recruited in the framework of the Hungarian General Practitioners' Morbidity Sentinel Stations Program (HMSSP). DNA samples of the population control individuals were used for haplotype analysis in AT and HHT founder mutations. Screening for the AT Bp3 mutation was done on 1000 samples, providing indirect evidence of the causative nature of the mutation. To prove the founder character of the AT

Bp3 and *ACVRL1* c.625+1 G> C mutations, haplotype analysis was performed on 200 and 50 control subjects, respectively.

4.3 Routine laboratory methods in antithrombin deficiency

Fasting blood samples were collected into 0.109 mol/L citrate vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ, USA) at least three months after the acute thrombotic episode and stored at -80°C until determination. Inherited thrombophilia (protein C and S deficiencies, APC resistance, dysfibrinogenaemia) was investigated by routine methods on a BCS-XP coagulometer (Siemens, Marburg, Germany). Presence of Factor V Leiden mutation (FVL) and prothrombin 20210G>A (FII G20210A) polymorphism were investigated on a LightCycler480 (Roche Diagnostics GmbH, Mannheim, Germany) by using real-time PCR and melting curve analysis.

For diagnosing AT deficiency hc-anti-FXa and p-anti-FXa (Labexpert Antithrombin H+P, Labexpert Ltd, Debrecen, Hungary; reference intervals 80-120% and 82-118%, respectively) were determined on a Siemens BCS-XP coagulometer [22]. AT antigen was measured by immunonephelometry (Siemens, N Antiserum to Human Antithrombin III, reference interval 0.19-0.31 g/L). AT activity, if sufficient plasma was available, was also determined by two commercially available functional assays based on FXa inhibition. Assay1 (Siemens, Innovance AT) uses human, while assay2 (HemosIL AT, Instrumentation Laboratory, MA, USA) uses bovine FXa as substrates and they also differ in the chromogenic substrate type (Z-D-Leu-Gly-Arg-ANBA-methylamide-acetate vs. S-2765 (N- α -Z-D-Arg-Gly-Arg-pNA•2HCl), final sample dilution (1:20 vs. 1:120), heparin concentration (1500 U/L vs. 3000 U/L) and in the dilution buffer (Tris-HCl, pH 8 vs. sodium-chloride).

4.4 Detection of causative mutations by fluorescent direct sequencing and MLPA analysis

Genomic DNA was isolated from peripheral whole blood by using the QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany). Sanger sequencing was executed to identify mutations in the exons, the flanking intronic regions and in the promoter using an ABI3130 Genetic Analyzer and Sequencing Analysis 5.4 software (Thermo Fisher Scientific, Carlsbad, CA, USA).

4.4.1 Antithrombin deficiency

If Sanger sequencing did not find causative mutations, multiplex ligation-dependent probe amplification (MLPA) was performed using SALSA MLPA KIT P227 (MRC-Holland,

Amsterdam, the Netherlands) using an ABI3130 Genetic Analyzer. The MLPA products were analyzed by GeneMapper Software 4.1 (Thermo Fisher Scientific).

4.4.2 Osler-Rendu-Weber disease

The oligonucleotides for the analysis of *ENG*, *ACVRL1* and *SMAD4* genes were designed by us. PCR reactions were performed in 40 cycles on a GeneAmp2700 PCR (Applied Biosystems, Foster City, CA, USA) instrument.

4.5 Investigation of the founder effect with polymorph genetic markers

The presence of the founder effect of AT Bp3 mutation was investigated on 63 AT Bp3 index patients (a total of 102 including family members) and on 200 samples from the HMAP control individuals. The possibility of the founder effect in the case of the *ACVRL1* c.625+1 G> C mutation was investigated on 5 probands (altogether 34 persons with family members included) and 50 samples from the HMAP control individuals.

For the testing of the SNPs and microsatellite markers, we designed the oligonucleotides. In each case, it was verified, that these oligonucleotides would amplify the corresponding DNA sequence region. Some of the SNPs were detected by FRET-based technique and others were detected by fluorescent direct sequencing; microsatellite markers were detected by fluorescence fragment analysis.

4.5.1 SNP detection using melting curve analysis and FRET detection

Melting curve analysis was used to detect the frequency of AT Bp3 mutation and the founder effect of this variant. Of the five polymorphisms investigated, three polymorphisms: rs677, rs1799876 and rs2227596 were tested in a single reaction, but the LightCycler®480 can also detect multiple fluorescent signals simultaneously. By overlapping the emission spectrum, we can compensate for "crosstalk" between the detection channels by color compensation. For the investigation of the rs941989 and rs2227612 polymorphisms, a multiplex reaction was performed and color compensation was used in the analysis. The two nonsense variants [p.Val327 (rs5877) and p.Glu337 (rs5878)] were also analyzed by fluorescence direct sequencing of exon 5 in the AT Bp3 mutation carriers and in 200 control subjects. The *SERPINC1* 5'-length polymorphism was detected by PCR following agarose gel electrophoresis.

After genotyping rs677, rs1799876, rs2227596, rs941989, rs2227612, rs5877 and rs5878 and 5'LP polymorph markers, haplotypes were generated using the software Haploview (<http://www.broadinstitute.org/haploview>).

In the region of q24.2-q25.2 of chromosome 1, four microsatellite markers (*SERPINC1*-Alu5 and Alu8, D1S196, D1S218) within the *SERPINC1* gene and around 12.8 cM; and F13A1-STR in the 6p25.3 region as a negative control were analyzed for AT Bp3 mutation carriers (n=102) and 200 control subjects.

4.5.2 Osler-Rendu-Weber disease

Melting curve analysis was used to screen for the *ACVRL1* c.625+1 G>C mutation in the sample of 50 control subject and the detection of the rs2071219 polymorphism in 34 family members and 50 control subjects was performed on a LightCycler®480 Instrument (Roche). Investigation of rs706815 and rs706816 polymorphisms in the intron region following the exon 9 of the *ACVRL1* gene was performed by fluorescence direct sequencing for family members and control subjects.

Five microsatellite markers of chromosome 12 were tested in *ACVRL1* c.625+1 G>C carriers and their family members (n=34) and the 50 control individuals. The fluorescently labeled oligonucleotides that were used in multiplex PCR reactions for the fragment analysis of STRs were self-designed.

4.6 Determination of pathogenicity of new mutations

In the case of novel mutation detection, it is essential to verify the pathogenicity of the genetic variation. For this purpose, direct and indirect methods can be used. Direct methods include biochemical characterization of the mutation *in vitro*, in the case of indirect methods one possibility is the *in silico* mutation prediction analysis.

4.6.1 Biochemical characterization of *SERPINC1* p.Leu205Pro new genetic variation

In the case of the index patient carrying the p.Leu205Pro mutation both the AT activity (58%) and the antigen level (0.19 g / L, corresponding to 76%) were decreased, so genetic testing was performed. At the time of genetic diagnosis the patient was 68 years old and several DVTs occurred earlier. In connection with the mutation, we had the opportunity to analyze a large, 4-generation family (n = 47 persons); where more people suffered from DVT. The detailed description of the biochemical and *in silico* characterization and the clinical history of the family is the main topic of another PhD thesis, only a part of the biochemical examinations of the mutation is presented here.

The cDNA clones ORF-NM_000488_pcDNA3.1(+) wild type AT (WT) and L205P mutant plasmids were purchased from ImaGenes GmbH (Berlin, Germany. Transient transfection in HEK293 cells was performed by using X-tremeGENE HP DNA Transfection

Reagent (Roche Diagnostics GmbH, Mannheim, Germany). The co-transfection of LacZ gene was also performed with pCMV Sport β -GAL plasmid (Invitrogen). After 48 h incubation, conditioned media was collected, the cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium dodecylsulfate and a protease inhibitor cocktail (Roche). The transfection efficiency was determined by FluoReporterlacZ/Galactosidase Quantitation Kit (Molecular Probes, Life Technologies) and the results were corrected accordingly.

Aliquots of the media and cell lysates were used for AT antigen determination by ELISA (AssayMax Human Antithrombin III ELISA Kit, Assaypro, St. Charles, MO). The AT antigen concentration values were measured on Labsystems iEMS Reader MF (Thermo Scientific) from three separate transfections.

Cell lysates and media were subjected to SDS-PAGE (10% gel) in non-reducing conditions and analyzed by Western blotting. AT was detected by goat anti-human AT antibody (Affinity Biologicals, Ancaster, Canada) using biotinylated, rabbit anti-goat IgG as secondary antibody. The immunoreaction was developed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), and visualized with 3,3'-diaminobenzidine (DAB) (Invitrogen) reagent. β -actin (8H10D10, CellSignaling Technology, Leiden, Netherlands) was used as internal control.

AT activity from the conditioned and concentrated supernatant of the transfected cells was determined by amidolytic assay in microtiter plate using LX Antithrombin Hc+P, FXa reagent (Labexpert Ltd., Debrecen, Hungary) with minor modifications. Both AT antigen (mg/mL) and AT activity (U/mL) were determined from the supernatant of the WT and P205 mutant AT in three independent experiments and specific activity was calculated and expressed as U/mg AT protein.

4.6.2 In silico prediction methods

The pathogenicity of the novel mutations was evaluated by three prediction methods, PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/index.shtml>), MutPred (<http://mutpred.mutdb.org>), and PhD-SNP (<http://snps.biofold.org/snps-and-go/snps-and-go.html>). For all the applied methods the score values between 0.5-1.0 mean possible/probable pathogenic mutations, while score values less than 0.5 predict likely benign mutations.

4.7 Statistical analysis

Kolmogorov-Smirnov test was performed to examine the normality of parameter distribution. Results of continuous variables were expressed as median and range. The significance of differences in continuous variables was tested by Mann-Whitney U test and Kruskal-Wallis test. χ^2 tests were used for differences in category frequencies. Kaplan-Meier survival curves were used to illustrate the difference in the time to the manifestation of first thrombotic event among the different AT deficiency types. A p-value of 0.05 or less was considered to indicate statistical significance. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 22.0), Chicago IL, USA.

5. RESULTS

5.1 Antithrombin deficiency

5.1.1 Genotype-phenotype correlations of antithrombin deficient patients

Between 2007 and 2016 we identified 31 different *SERPINC1* mutations (n=22 type I and n=9 type II) with high mutation detection rate (98%) in 156 index patients (n=246 with family members). Among the 31 mutations 11 novel ones were detected (36%). Most of the index patients carried type II.HBS mutations (75.6%) due to the high frequency of the founder AT Bp3 (86% out of type II.HBS). AT Bp3 was registered in homozygous and heterozygous forms, while all other *SERPINC1* mutations were detected only in heterozygous state. AT Basel and AT Padua I were detected in 5 and in 11 families, respectively. Each type II.RS mutation (AT Stockholm, AT Glasgow and p.Ile386Thr) was registered in one family. AT Torino and AT Budapest 5 (type II.PE) were registered in one family and in five families, respectively. Type I deficiency, regardless of the mutation was detected in 25 families.

Only in the case of three patients having borderline hc-anti-FXa AT activity gave both Sanger sequencing and MLPA analysis a negative result. The first patient had 79%, the second patient had 75% and the third patient had 76% hc-anti-FXa activity. All of them had borderline normal AT antigen concentration (0.22, 0.21 and 0.22 g/L, respectively) and acquired AT deficiency was not excluded.

The frequency of VTE was significantly higher in type I AT deficiency (66% vs. 49%, $p=0.015$) as compared to all type II heterozygotes. Comparing type I to type II.HBS heterozygotes, the difference in the frequency of VTE was even higher (66% vs. 41.8%, $p=0.003$). In contrast, the frequency of pregnancy complications was significantly higher in type II.HBS heterozygotes than in type I patients (2.1% vs. 7.1%, $p=0.046$). Pregnancy complication was registered only in one woman with type I deficiency carrying the p.Arg164* mutation. Among type I deficient patients ATE (myocardial infarction, MI) was registered only in one case with AT Wobble mutation and having several risk factors for MI (smoking, hypertension, hyperlipidaemia). No ATE or pregnancy complications were registered among type II.RS (n=3) and II.PE (n=12) individuals.

The time to the first manifestation of VTE and of any thrombotic event (including VTE, ATE and pregnancy complications) was compared between type I, type II.HBS heterozygotes and AT Bp3 homozygotes. When VTE was considered as clinical outcome, the age was significantly lower in type I patients as compared to type II.HBS heterozygotes (median age of type I AT deficient patients: 34 year, 95%CI: 30-38 years vs. median age of type II.HBS heterozygotes: 46 year, 95%CI: 33-59 years; $p=0.002$), and it was significantly higher when compared to AT Bp3 homozygotes (median age of AT Bp3 homozygotes: 15

year, 95%CI: 12-18 years; $p<0.001$). When the clinical outcome was considered as a composite of VTE, ATE and pregnancy complications, the time of the first manifestation did not differ significantly between type I and type II.HBS heterozygotes (median age of type I AT deficient patients: 34 year, 95%CI: 30-38 years vs. median age of type II.HBS heterozygotes: 40 year, 95%CI: 38-42 years $p=0.064$). AT Bp3 homozygosity was more severe than type I (median age of AT Bp3 homozygotes: 15 year, 95%CI: 12-18 years; $p<0.001$) in this aspect. Moreover, the frequency of proximal thrombosis was higher in AT Bp3 homozygosity as compared to type I deficiency (62.5% vs. 14.3%, $p=0.002$), while there was no difference between type I and type II.HBS heterozygotes.

There was no significant difference in the ratio of patients with FVL or FIIG20210A mutations among the different AT deficient groups and no other inherited thrombophilia was registered in any of our patients. There were no differences in the ratio of patients with acquired risk factors or provoking factors. Antiphospholipid syndrome was not found in our patients. The number of patients with congenital vascular anomaly was low (5%) in all groups.

Due to the high number of patients with type II.HBS deficiency we were able to compare the clinical characteristics among the different subgroups. As it was expected, the ratio of symptomatic patients was the highest (92.3%) in AT Bp3 homozygotes; it was significantly higher than in AT Bp3 heterozygotes (56.6%, $p=0.001$) and in AT Padua I (53.3%, $p=0.006$). VTE was more frequent in patients with AT Bp3 mutation as compared to AT Padua I and AT Basel (AT Padua I vs. AT Bp3 heterozygotes 20% vs. 48.7%, $p=0.041$; AT Padua I vs. AT Bp3 homozygotes 20% vs. 92.3%, $p<0.001$; AT Basel vs. AT Bp3 homozygotes 14.3% vs. 92.3%, $p<0.001$). The frequency of VTE was the highest in AT Bp3 homozygotes (AT Bp3 heterozygotes vs. AT Bp3 homozygotes $p<0.001$). ATE was the most frequent in AT Basel (AT Basel vs. AT Bp3 heterozygotes 42.9% vs. 10.5%, $p=0.046$; AT Basel vs. AT Bp3 homozygotes 42.9% vs. 0%, $p=0.006$). Pregnancy complications were the most frequent in AT Padua I (26.7%); however, the difference was not statistically significant among the different type II.HBS groups. The number of patients with proximal localization of thrombus was significantly higher in AT Bp3 homozygotes than in AT Bp3 heterozygotes ($p=0.001$). There was no significant difference in the presentation of additional thrombosis risk factors among the different type II.HBS groups.

The time to the first VTE was not different between AT Basel and AT Padua I (median age of AT Basel: 51 years, 95%CI: 41-62 years vs. median age of AT Padua I: 62 years, 95%CI: 53-71 years; $p=0.982$) and between AT Basel and AT Bp3 heterozygotes (median age of AT Bp3 heterozygotes: 40 years, 95%CI: 35-45 years; $p=0.095$). VTE

occurred significantly earlier in AT Bp3 heterozygotes than in AT Padua I ($p=0.010$). AT Bp3 homozygotes were the youngest at the time of the first VTE (median age of AT Bp3 homozygotes: 15 year, 95%CI: 12-18 years; AT Bp3 homozygotes vs. AT Basel $p=0.002$; AT Bp3 homozygotes vs. AT Padua I and vs. AT Bp3 heterozygotes $p<0.001$). When the composite outcome was compared, the time to the first manifestation did not differ significantly between AT Basel and AT Padua I (median age of AT Basel: 49 year, 95%CI: 18-80 years vs. median age of AT Padua I: 43 year, 95%CI: 33-53 years; $p=0.459$), between AT Bp3 heterozygotes and AT Basel (median age of AT Bp3 heterozygotes: 40 year, 95%CI: 38-42 years; $p=0.997$) and between AT Bp3 heterozygotes and AT Padua I ($p=0.130$). The time to the first manifestation was significantly lower in AT Bp3 homozygotes as compared to other type II.HBS groups (AT Bp3 homozygosity vs. AT Bp3 heterozygosity $p<0.001$, AT Bp3 homozygosity vs. AT Padua I $p<0.001$ and AT Bp3 homozygosity vs. AT Basel $p=0.018$).

The detection of a large number of AT Bp3 homozygous carriers (the largest number of patients in the literature) made it possible to compare to AT Bp3 heterozygotes in several ways. Among the AT Bp3 homozygotes, 13 severe or unusual localization DVTs occurred. Seven patients had iliofemoral and two patients had vena cava inferior thrombosis. In one patient both vena cava inferior and vesicular thrombosis occurred. Mesenteric thrombosis was present in one patient and tricuspidal valve thrombosis was present in one patient as well. Among the AT Bp3 homozygotes, only 2 asymptomatic individuals were registered. Among the AT Bp3 heterozygotes there were 8 cases of arterial events (4 patients with only ATE, 4 with ATE + VTE), in contrast to nobody among the homozygotes. Asymptomatic AT Bp3 heterozygotes were very young at the time of data analysis (median: 21 years, range: 2-53 years), only 2 cases carried other genetic risk factor, the FVL mutation, in heterozygous form. Comparing the TE risk, between AT Bp3 homozygotes and heterozygotes, AT Bp3 homozygotes seem to have a 14.06 times higher chance (95% confidence intervals 3.10-63.74) of developing thrombotic events than heterozygotes.

5.1.2 Determination of founder effect in patients with antithrombin deficiency

The very high frequency of AT Bp3 in our study population suggested a founder effect. AT Bp3 was absent in 1,000 healthy individuals recruited by the HMSSP program. The MAF values of the examined SNPs and the 5'LP were lower in the AT Bp3 carriers as compared to the values observed in the general population and to the data available for the European population. The minor alleles were absent in the homozygous AT Bp3 patients in case of all SNPs and 5'LP. The rs5877, rs5878, rs1799876 and rs941989 showed linked inheritance in

all examined individuals. Two additional SNP's showed linked inheritance (rs677 and rs2227596). Due to these relations investigation of rs5877, rs5878, rs1799876 and rs677 was not mandatory to perform in the general population to draw conclusions.

It was demonstrated that in the case of all AT Bp3 carriers the pathogenic „T” allele was associated with one haplotype. The normal „C” allele was associated with different haplotypes both in AT Bp3 carriers and controls. AT Bp3 homozygous patients shared one distinct Alu5 and Alu8 repeat number variations (ATT)₆ and (ATT)₁₅, respectively. In controls repeat numbers 6 and 8 were detected for Alu5 and different STR repeat numbers were seen between 1 and 19 for Alu8. The STR marker closer to *SERPINC1* showed bi-allelic distribution in AT Bp3 homozygotes carrying (AC)₂₄ and (AC)₂₅. The distribution of the population control group was heterogeneous; different repeat numbers between (AC)₁₉ and (AC)₃₃ were observed. STR marker D1S196, which locates proximal and farther to *SERPINC1* showed high degree of variability both in AT Bp3 homozygotes and in controls. As a negative control, one extra-chromosomal STR marker (F13A1-STR) was also screened and showed heterogeneity; the same repeat numbers, (AAAG)₃₋₂₀ were found in controls and AT Bp3 patients. Where it was possible informative family trees were also generated.

5.1.3 Novel *SERPINC1* variants

Genotype-phenotype correlations in patients with novel SERPINC1 mutations

Among our 11 novel AT variants all mutations except for p.Pro461Thr showed a laboratory phenotype of type I deficiency.

Among the novel variants mutations leading to early stop codon are carried by 7 individuals belonging to 5 families and all but one patient (age 37 year) suffered from early DVT. No additional risk factors were detected in any of them. One individual carrying the p.Lys171Valfs*16 mutation, one patient with p.Arg79Profs*34 and one patient with p.Leu270Argfs*14 mutation had two or more episodes of thrombosis, the latter patient suffered 4 episodes of thrombosis. Patient carrying the p.Gly456delinsAlaThr mutation had two DVT episodes and provoking factor was registered only at the second onset. Among the missense variants the p.Leu205Pro mutation was detected in a large family with 11 carriers. Two carriers of the p.Asn450Ile mutation already had DVT and among them the patient having two episodes of thrombosis is also a carrier of the FVL mutation.

The novel p.Pro461Thr mutation is carried by one individual, she was the oldest at the time of DVT diagnosis (62 year) and it is worthy of mentioning that the BMI of this female is 32.

In silico prediction of novel missense mutations

Sequential alignment for AT in human and in different species showed that all the novel missense mutations affected conservative regions. In the *in silico* prediction models the AT Bp3 mutation (p.Leu131Phe) and the AT Cambridge II (p.Ala416Ser) mutation were used as positive controls, since their pathogenicity had been confirmed by different *in vitro* studies before. All but the p.Leu131Phe mutations were predicted as definitely pathogenic by the methods. Among the novel missense variants the p.Leu205Pro mutation has been already characterized by our group

Investigating pathogenicity of a new mutation by biochemical methods

Wild type AT appeared as a clear band in the conditioned media of HEK cells at 58 kDa, however, only a faint band of Pro205 AT could be visualized. Both WT and mutant AT were demonstrated as equally intense bands in the lysates of transfected HEK cells around 58 kDa. As it was expected no signal from mock transfection could be detected. The positive control band represented AT from the pooled plasma of 5 healthy individuals and appeared as a single band.

AT antigen concentration was determined by ELISA in four independent experiments (i.e. four independent transfections of HEK cells, AT was measured 48 h after each transfection in duplicates) and corrected for transfection efficiency. AT concentration was 2.33 ± 0.76 $\mu\text{g/mL}$ in the media of the WT cells, while it was only 0.56 ± 0.43 $\mu\text{g/mL}$ in the media of cells containing Pro205 mutant AT. In the cell lysates – in accordance with the results of the Western blotting - AT concentration was 2.83 ± 1.40 $\mu\text{g/mg}$ and 2.86 ± 1.10 $\mu\text{g/mg}$ protein for cells transfected with WT and mutant plasmids, respectively.

For determining the specific activity of recombinant AT both AT activity and antigen levels were measured in the conditioned and concentrated media of cells expressing WT AT and Pro205; all measurements were executed in samples obtained from three independent transfections. AT antigen concentration in the WT media was 0.21 ± 0.06 mg/mL and it was 0.06 ± 0.01 mg/mL in the Pro205 media. By performing the amidolytic assay AT activity values were 1.576 ± 0.001 U/mL and 0.221 ± 0.058 U/mL in the WT and mutant media, respectively. The average specific activity (i.e. the activity related to one mg AT protein) obtained in the three independent experiments of Pro205 was 3.94 ± 0.95 U/mg , while it was 7.79 ± 2.10 U/mg in the case of the WT AT.

5.1.4 Pediatric patients with AT deficiency

In our cohort 32 patients had the first thrombotic episode in childhood (i.e. before the age of 18). Most of them were of type II.HBS mutants (n=25) and the majority of children (n=18) were homozygous carriers of AT Bp3. There were two peaks of age (0-1 years and 12-18 years) in the time of first thrombotic episode.

All but one infants (i.e. 0-1 years, n=7) with thrombosis were AT Bp3 homozygotes. Only one of them was a heterozygous carrier of FVL and hereditary vascular anomaly was diagnosed only in 2 children. The infant with AT Truro had sinus sagittal thrombosis and cerebral vein hypoplasia was diagnosed as well.

There were 20 patients older than 12 years of age at the time of first thrombotic episode and among them n=15 were carriers of AT Bp3 and provoking factor could be explored only in 4 patients. No additional hereditary or acquired thrombosis risk factors were found in the background. One patient with AT Basel suffered from ischemic stroke and MI and 2 patients with AT Bp3 heterozygosity had ischemic stroke.

5.1.5 Laboratory phenotype of patients with *SERPINC1* mutations

Hc-anti-FXa AT activity and p-anti-FXa activity were low and correlated well in type I deficient patients. AT antigen concentrations were below the lower limit of the reference interval in all cases.

Both hc-anti-FXa activity and p-anti-FXa AT activities were low in all II.RS and II.PE patients and AT antigen levels were all normal. It is interesting, that in type II.HBS patients AT levels were different according to the specific mutations. In theory, hc-anti-FXa activity is low in type II.HBS deficiency, while p-anti-FXa activity is normal and the high p-anti-FXa/hc-anti-FXa AT activity ratio discriminates well between type II.HBS and other type II AT deficiencies. In our study the hc-anti-FXa assay that was used for diagnosis, gave low AT activity in all type II.HBS cases (100% sensitivity). In the case of AT Basel and AT Padua I the p-anti-FXa activity and AT antigen were within the reference interval in all patients (as expected); the median of p-anti-FXa/hc-anti-FXa ratio was 1.66; range 1.53-2.05 and it was 1.89; range 1.71-2.10, respectively. However, low p-anti-FXa activity and AT antigen concentration were detected in some patients with AT Bp3 (especially homozygotes). The median p-anti-FXa/hc-anti-FXa ratio values in heterozygotes and homozygotes were 1.51 (range 1.28-2.11) and 5.60 (range 2.88-8.85), respectively.

The functional assay used for diagnosis was compared to 2 similar (i.e. FXa-based in the presence of heparin) commercially available assays. The assays gave similar results in type II.RS and type II.PE patients. In type II.HBS AT Padua I and Basel AT activity values

differed markedly between assay 1 and 2. While assay 1 and our assay gave low AT activity in all cases, assay 2 did not recognize these mutants showing normal AT activity for all patients. AT activity values were decreased in AT Bp3 homozygotes with all assays; however, the results obtained by assay 2 were significantly higher than those in the other tests, suggesting heterozygous state. The sensitivity of Assay 2 for AT Bp3 heterozygosity was only 44%.

As it was suggested by our findings and by earlier studies, the substrate type (i.e. anti-FIIa or anti-FXa assay) is not the only factor that influences the sensitivity of functional AT assays. To investigate the effect of different heparin concentrations and pH on assay sensitivity we performed a pilot experiment using our diagnostic assay. Upon increasing the heparin concentration in the assay all AT activity values increased and in one sample with AT Padua I it reached the lower limit of the reference interval. AT activity values further increased, reaching or exceeding the lower limit of the reference interval in 2 AT Basel and in 1 AT Padua I samples by changing the assay conditions to pH 7.4. When the heparin concentration of the original assay increased by 8-fold all AT Basel and Padua I samples gave normal AT activity results. AT activity values of AT Bp3 samples did not increase further.

5.2 Osler-Rendu-Weber disease

5.2.1 Diagnosis of patients with Osler-Rendu-Weber disease

Beyond the five probands, a total of 34 at-risk individuals from the five families were available for genetic screening. Physical examination was performed in all of them. When the family history was recorded, we investigated if there were other persons in the family who had nosebleeds, TA and/or AVMs. The presence of AVM was determined by various imaging methods (brain contrast-mediated MR, lung and liver CT scan).

In the genetic background of the 34 HHT suspected patient (5 probands and 29 at-risk family members) from the Northeastern region of Hungary we could not detect mutations in the *ENG* gene by fluorescence direct sequencing. Continuing the examinations in the *ACVRL1* gene, an unknown genetic variation, the c.625+1 G>C splice site mutation was observed in heterozygous form in 19 individuals. Physical examination was performed in 18 cases of mutation carriers, and 13 individuals had nose bleedings (25 ± 12.6 years) and 14 patients were registered with TAs (39.5 ± 13.7 years). Four cases required transfusion due to severe nosebleeds. The presence of AVM was investigated in 11 adults, 4 had AVMs in the liver. Also in 4 cases, non-bleeding GI and duodenal TAs have been observed. Brain and pulmonary AVMs did not occur in this patient group.

In the case of the 5 probands, four of them were from southern part of Heves county and one from the adjacent Jász-Nagykun-Szolnok county. They did not have any relationship with each other, but a match between the names of their ancestors was found. In families, living in close proximity, carrying the same splice site mutation (*ACVRL1* c.625+1 G>C), the relationship and a founder effect were suspected. To clarify these, further genetic studies were performed in the 5 probands, in 22 relatives and in 50 controls (who did not carry the *ACVRL1* c.625+1 G>C mutation), and HHT related family trees were also generated.

5.2.2 Determination of founder effect in patients with Osler-Rendu-Weber disease

In the haplotype analysis, 3 intronic polymorph markers were genotyped in the 15 HHT patients and in the 50 control individuals.

Allele frequency values in the control group for all three SNPs were similar to HapMap's Caucasian population data (data for 2015). In contrast, the allele frequency values of HHT patients differed significantly from HapMap and thus from the allele frequency values of the control group. Statistical analysis was not done due to the low number of cases.

During the fluorescence fragment analysis of the microsatellite markers, one intragenic STR (D12S1677), 2 proximally localized (D12S85, D12S196) and 2 distally localized (D12S1712, D12S270) STRs from *ACVRL1* gene were detected. The D12S85 proximal farther marker exists in the 80% of patients with 12 repeat number variation, while in the control group, the recurrent repeat number variations varied between 11 and 38 repeats. The D12S196 marker, which is also proximal but closer to the gene, the repeat number 10 and 7 are the most recurrent in the patients, and the repeat numbers 10 and 9 in the control group are the most common. For the D12S1677 intragenic marker, 60% of the patients had 20 repeat number variations, nearly 25% of them had the 21 repeat numbers; while in the control group there were varied repeat numbers. In the case of D12S1712 microsatellite distal and closer to the gene, more than 60% of the patients and the majority of the control subjects had the 16 repeat numbers. The distal and farther D12S270 marker showed a varying number of repeat numbers in both patients and controls.

By analysing the families of the 5 probands, we made a family tree with the registered haplotypes. In mutation carrier individuals, the same haplotype was observed for the mutant "C" allele.

A geneologist discovered the ancestry of the 5 probands and a common ancestor from whom the *ACVRL1* c.625+1 G>C mutation was derived. Finally, a married couple was found whose marriage dates back to 1779, approximately 30 kilometers far from the present-day residence of their descendants.

6. DISCUSSION

6.1 Genetic studies in antithrombin deficiency and Osler-Rendu-Weber disease; founder mutations

One of our aims was to detect the genetic background and to find common mutations in AT deficient patients and Osler-Rendu-Weber patients from Hungary and the surrounding geographical regions.

AT is the key regulator of coagulation. The inherited AT deficiency is a heterogeneous disease, it can classify in type I (quantitative) and type II (qualitative) deficiency. In our AT deficient population 31 different *SERPINC1* mutations were detected by fluorescence direct sequencing and MLPA analysis, of which 11 were novel variants. In this AT deficient patient group, the II.HBS subtype was the most common.

The Osler-Rendu-Weber disease (or hereditary hemorrhagic telangiectasia, HHT) is an autosomal dominant abnormality, characterized by nosebleeds, TAs and AVMs. Diagnosis of the disease is based on the 4 Curaçao criteria established in 1999. The *ENG* and *ACVRL1* gene mutations are in the background of HHT. No mutations were found by direct sequencing of *ENG* gene in the case of the 5 HHT suspected probands from the Northeast region of Hungary, however, in the *ACVRL1* gene all 5 patients were identified with the same genetic variant (*ACVRL1* c.625+1 G>C), which was not reported so far in the literature. This splicing variant was also registered in family members of 5 probands whom fulfilled at least 2 Curaçao criteria.

6.1.1 Significance and appearance of founder mutations in antithrombin deficiency, other thrombophilias and in Osler-Rendu-Weber disease

Founder mutations reduce genetic diversity in a small population, and changes occur in allelic frequencies as a result of genetic drifts. To verify the presence of founder mutations, linkage analysis is performed. Polymorphic genetic markers (microsatellite) allow a thorough examination of genetic variance. The founder effect can be verified if the causative mutation in the background of the disease shows close correlation with the specific haplotype with the tested polymorphic markers. Detection of founder mutations in a population is useful in many ways. On one hand, due to the founder mutation, we can raise a much more efficient diagnostic strategy for the affected patient groups. The presence of founder mutations increases mutation detection rates in case of a particular genetic disease. In addition to a faster and more accurate diagnosis, it is also important to note that similar clinical phenotypes are associated with carriership of the same mutation, making easier to plan prevention and implement therapeutic strategies in these patients.

The AT deficient patients in Hungary carried the AT Bp3 (p.Leu131Phe) mutation in a high proportion. Practically all patients with this mutation described to date were of Central Eastern European origin. Beside the occurrence of VTE, arterial thrombotic events and pregnancy complications were also described in some of these patients. Olds et al. based on the analysis of six genetic markers, including STRs Alu5 and Alu8 suggested a founder effect; however only five families carrying ATBp3 were eligible for that study. In our study we had the possibility to confirm the existence of a founder effect and described the genotype-phenotype relations in the highest number of ATBp3 patients reported so far. Altogether we used twelve genetic markers (7 SNPs, one 5'LP and 4 STRs), including all the markers that were investigated in Olds's study and six additional ones. Our investigation in 102 carriers of ATBp3 and 200 healthy controls undoubtedly confirmed the founder effect. In the case of all ATBp3 carriers the pathogenic „T” allele was associated with only one haplotype. The normal „C” allele was associated with different haplotypes both in ATBp3 carriers and controls. It is interesting that the AT mutation spectrum of the Finnish population differs from that of the Hungarian and no ATBp3 was detected in the study of Puurunen et al, although the two nations are considered to be in relation. Inherited type II AT deficiency is caused, in the great majority (88%) of Finns, by a single founder mutation, AT Basel (p.Pro73Leu), however, in the Hungarian population its frequency is only 4%. In contrast, while AT Bp3 is 81% frequent in Hungarian type II AT deficient population, this mutation does not occur in Finnish patients. Based on these, it is possible that the AT Bp3 mutation was generated or got into the Hungarian population after separation of the two ethnic groups. Estimates of the time of founding have already been carried out in our working group, according to which the XVII. century seems to be the most probable, but further clarifying tests are needed before publication. AT Cambridge II is frequent in the British population; the mutation has a prevalence around 0.5-2.0% in French, Spanish and German VTE patients. AT Cambridge II, however was not detected in other populations like the Hungarian or populations from Southern China. Investigating the possibility of the founder effect in the case of AT Cambridge II 29 out of 31 patients carried the same haplotypes except for an STR (ATT)₈ where 4 types of repeat number variations were found. They concluded that 4 unrelated person could be the founders of the AT Cambridge II mutation. AT Cambridge II can be associated with both venous and arterial thrombotic events. Olds et al. studied five p.Arg161* *SERPINC1* mutation carrier families, in which the possibility of a founder mutation was also raised. Linkage analysis was performed in the case of AT Hamilton (p.Ala414Thr) and AT Amiens (p.Arg79Cys) mutations. In both cases, (ATT)₈ has been characterized by a typical frequency of (ATT)₁₇ in AT Hamilton and ATT₍₁₈₎ in AT Amiens

carriers. Both AT Hamilton and AT Amiens mutations associate with an increased risk of venous thrombosis. Finally, the most extensive genetic study was carried out by our group on the largest AT deficient population to clarify the founder effect of a *SERPINC1* mutation.

The 5 patients with Osler-Rendu-Weber disease were from a close geographic region, the identity between their ancestors and the same mutation (*ACVRL1* c.625+1 G>C) raised that we found a founder mutation. To confirm this, 8 polymorphic genetic markers were studied in the q13.11 - q13.13 region of chromosome 12. For the haplotype analysis, three SNPs (rs2071219, rs706815, rs706816) and a microsatellite marker (D12S1677) within the gene, two proximal located (D12S85, D12S2196) and two distal located (D12S1712, D12S270) STRs from the *ACVRL1* gene were analyzed. During the haplotype analysis we detected the same haplotype associated with the mutant "C" allele. The studied 8 polymorphic markers were located on the same chromosome, close to each other and represent linked inheritance. Recombination was not observed on the mutation carrier allele. This suggests a smaller number of meiosis in which crossing over could have occurred, placing the occurrence of the mutation in the not so distant past. Tracing back the genealogy to the couple, whose wedding was in 1779, does not necessarily mean that they were the founders of the mutation. HHT founder mutations have been found in almost all parts of the world, including the Netherlands Antilles, France, Italy, Denmark and Norway.

6.1.2 Mutation detection rate in antithrombin deficiency and Osler-Rendu-Weber disease

The high mutation detection rate (98%) that was observed in our study may be caused mainly by the high prevalence of the founder ATBp3 mutation. In other populations the detection rate is between 69-83%. In the background of lower mutation detection rates in patients with low AT levels an aberrant N-glycosylation was hypothesized, or mutations in the regulatory sequences of *SERPINC1* were suggested. An algorithm for laboratory diagnosis of AT deficiency was established, which is based on the determination of hc-anti-FXa, p-anti-FXa, AT antigen and molecular genetic testing. This hemostasis diagnostic algorithm we developed is 100% sensitive and specific.

Diagnosing the patients with Osler-Rendu-Weber disease from Northeastern Hungary, the mutation detection rate was 81.3% (unpublished data). The mutation detection ratio is only 36.7% in patients from another regions of the country. In our opinion, the reason behind this discrepancy might be explained, that while in the Northeastern region the diagnosis of the disease was carried out strictly in accordance with the Curaçao criteria, the diagnosis of HHT was not always sufficiently substantiated in the case of patients from other parts of the

country. For this reason, we suggest the preparation of a professional recommendation, which could be a guideline for diagnosing of HHT. The very high (81.3%) mutation detection rate in the Northeastern region is also the consequence of the founder mutation (*ACVRL1* c.625+1 G>C). In general, taking into account the 4 Curaçao criteria, in approximately 75% of HHT cases either the *ENG* or the *ACVRL1* mutation can be detected. The detection rate is above 85% when the 4 criteria are present simultaneously.

6.1.3 Novel mutations in antithrombin deficiency and Osler-Rendu-Weber disease

Detecting novel mutations in AT deficiency and in HHT patients is common, as there are no mutation hot spots, and in the case of HHT more than one gene is affected.

Among our 11 novel AT variants all mutations except for p.Pro461Thr showed a laboratory phenotype of type I deficiency. The p.Pro461Thr mutation is suggested as a type II_{PE} variant based on our laboratory results and on a publication, in which the p.Pro461Leu was described in a type II_{PE} deficiency.

The *ACVRL1* c.625+1 G>C mutation was not included in any mutation databases or publications, therefore it was considered as a novel one.

In the near future, when genetic diagnosis with next generation sequencing (NGS) will be more affordable, the diagnosis will be easier than functional diagnosis, making possible to identify many (novel) abnormalities. When detecting a novel mutation, it is crucial to evaluate the pathogenicity of the respective mutation. There are indirect and direct pieces of evidence for verifying the pathogenicity of a novel mutation. Indirect confirmation includes the demonstration of conservatism; in our case, *SERPINC1* novel missense mutations occurred in conserved regions. Further evidence of pathogenicity can be found using *in silico* methods (software), which are widely used today. During this work, we used the PolyPhen2, MutPred and PhD_SNP software to test the *SERPINC1* missense mutations we found, to determine which could be considered as pathogenic. We could also perform segregation analysis in large-scale families to describe the appearance of a specific phenotype and to clarify certain gene effects. In our own study, both in AT deficiency and in HHT patients we had the opportunity to study large families with multiple generations (p.Leu205Pro in the *SERPINC1* gene and *ACVRL1* c.625+1 G>C). It can be considered as indirect evidence if we can't detect the novel genetic variant in at least 100 healthy persons' alleles. We used the latter method for *SERPINC1* mutations and in the case of *ACVRL1* c.625+1 G>C mutation.

The main evidence of pathogenicity of a mutation (direct evidence) is provided by *in vitro* biochemical analysis when the quantitative and qualitative consequences of the mutation in recombinant systems are investigated. Of the 11 novel AT mutations found by our working

group, we have completed the molecular characterization in one of them. The p.Leu205Pro mutation is carried by several members of a large family who had several thrombotic events. The Pro205 mutant protein was detected in the lysate of cells similar to that of WT AT, and protein synthesis was not affected. In contrast, only a small amount of mutant protein expressed into the supernatant of the cells, suggesting a secretion defect. The specific activity of the Pro205 protein is also decreased in contrast to WT AT. In summary, the results suggest, that the biochemical consequence of this mutation is rather complex (quantitative deficiency due to impaired secretion and functional dysfunction).

6.2 Genotype-clinical phenotype relationships in antithrombin deficiency and Osler-Rendu-Weber disease

6.2.1 Clinical manifestations of antithrombin deficiency

From the point of view of clinical manifestations type I AT deficiency is rather homogeneous, while type IIHBS seems to be a heterogeneous group.

Concerning the clinical consequences of type I deficiency AT mutations, in general it was associated with a severe venous thrombotic phenotype in our cohort, except for AT Wobble, in which case no VTE but MI was registered. AT Truro was considered as type IIHBS variant based on an *in silico* experiment, however, the laboratory phenotype observed in our study and by others suggests a quantitative defect. The biochemical consequences of this mutation therefore would be interesting to study.

Type II.HBS is an interesting group of AT deficiency, where the clinical and laboratory phenotypes are more heterogeneous and dependent on the specific mutation. AT Bp3, AT Padua I and AT Basel associated with not only VTE but also with ATE and pregnancy complications with different frequencies as well. When the time to the first manifestation of VTE was considered as a measure of clinical severity, in our study, it is to be highlighted that type I AT deficiency is more severe than heterozygous II.HBS deficiency, however AT Bp3 homozygosity is more severe than type I AT deficiency in this aspect. Within type II.HBS group AT Bp3 homozygosity is the most severe and ATBp3 heterozygosity is significantly more severe than AT Padua I. AT Basel and AT Padua I are not significantly different from each other concerning the time to the first manifestation of VTE. When the time to first manifestation of a composite clinical endpoint, including not only VTE but also ATE and pregnancy complications, is taken into consideration, the severity of type I and type II.HBS heterozygotes do not differ significantly. AT Bp3 homozygosity is more severe than type I and type II.HBS heterozygous AT deficiency in this aspect. AT Basel is rather associated with ATE; the highest number of pregnancy complications is detected in

AT Padua I. It is interesting, that no homozygous patients with AT Basel and Padua I were found in our study, and screening the literature revealed that no such patients had been described elsewhere. It is very likely, that at least one reason for not finding homozygous AT Basel or Padua I patients in our study is the relative rarity of these mutations comparing to the frequency of AT Bp3. It is also possible, that – due to their severity - AT Basel and Padua I are lethal mutants in homozygous form.

6.2.2 Manifestation of antithrombin deficiency in childhood

According to our knowledge, our study group collected one of the highest number of pediatric AT deficient symptomatic patients to date, among them the frequency of type II.HBS was the most prevalent. These findings highlight the importance of AT deficiency screening in unprovoked pediatric thrombosis cases especially in populations, where the prevalence of type II.HBS AT deficiency is high.

6.2.3 Therapeutic aspects of antithrombin deficiency

In our cohort, long-term anticoagulation is the practice for the therapy of children, and in most cases this is the protocol for treatment of adults. After thrombosis, the duration of anticoagulation as secondary prevention is not consistent. AT deficiency is a serious thrombophilia and the prevention strategy of AT deficient patients may differ from the strategy used in other thrombophilias. An international prospective database would be needed to provide a well-founded recommendation on therapy. Another important aspect is the prevention strategy of asymptomatic mutation carriers; to determine when and which preventative forms are needed. In our investigations, 29 under the age of twenty and 64 under the age of fifty years asymptomatic family members were registered. If we register asymptomatic mutation carrier family members in the targeted genetic screening of the proband's family, we have the option of primary prevention in cases of high thrombosis risk. According to some recommendations, oral contraceptives should not be prescribed in their case.

6.2.4 Clinical manifestations of Osler-Rendu-Weber disease

In our own studies, the HHT occurred with varied phenotypes in families with *ACVRL1* c.625+1 G>C mutation. Two 34 years old patients had no nosebleeds until the time of diagnosis. In the mutation carriers, the appearance of TAs on face, lips, tongue and hands was relatively late, at the end of their 40s. Lung and cerebral AVMs did not occur. Four patients were diagnosed with liver AVMs. The importance of family screening is also emphasized in

HHT, because monitoring of genetic mutation in symptom-free family members is required to prevent serious bleedings and its treatment as soon as possible.

6.3 Laboratory aspects for antithrombin deficiency

For laboratory diagnosis of AT deficiency the first-line test is a chromogenic functional assay, in which the inhibition of thrombin (FIIa) or FXa in the presence of heparin (heparin cofactor activity, hc-anti-FIIa or hc-anti-FXa) is measured.

It is interesting that there are big differences in the sensitivity even among hc-anti-FXa assays to type II.HBS AT deficiency. It is to be noted, that type II.HBS AT deficiency may be under-diagnosed by some commercially available functional assays. Based on the results of our pilot experiment, it is suggested that the high heparin concentration and perhaps the lower ionic strength are major factors that decrease the sensitivity of the assays to type II.HBS deficiency, especially to AT Basel and Padua I. Our results strengthen the hypothesis that type II.HBS deficiency is a heterogeneous group with different strength of heparin binding according to the specific mutations, and consequently leading to different behavior in the functional assays. Assay modifications that were tested, had less effect on AT activity values of AT Bp3 samples, and taken together with the low AT activity of AT Bp3 homozygotes, suggests a more complex consequence of this mutation than being just a heparin-binding defect.

New results of the PhD thesis

- The highest mutation detection rate was reported in the literature during the genetic examination of AT deficient patients and their family members; 31 different, amongst these 11 novel mutations were identified in the *SERPINC1* gene.
- We have found that the AT Bp3 mutation (p.Leu131Phe) belonging to the II.HBS subtype is the most common mutation in Hungarian AT deficient population and we have confirmed with polymorphic genetic markers a founder effect in the background.
- We have found that AT deficiency is a heterogeneous group from the point of view of clinical manifestation; and even the II.HBS subtype itself is heterogeneous. While type I deficiency is associated with severe venous thrombosis, the II.HBS subtype in heterozygous form is milder and not exclusively associated with venous thrombosis. AT Basel often associated with ATE, AT Padua I was more common in pregnancy complications. AT Bp3 homozygous patients had the most severe clinical form where venous thrombosis often manifested in early childhood. For non-provoked thrombosis in childhood, it is highly recommended to investigate AT deficiency.
- We have found that type II. AT deficiency is heterogeneous from the point of view of laboratory diagnosis. We demonstrated that the sensitivity of the functional heparin cofactor assay is not exclusively dependent on the type of enzyme (thrombin or FXa), but also influenced by other factors.. Within the II.HBS type, HemosIL AT showed a 44% sensitivity to AT Bp 3 and was completely insensitive to AT Basel and AT Padua I mutations. Heparin concentration and pH are important factors for the sensitivity of FXa-based tests.
- In the case of the 11 novel *SERPINC1* mutations, we have now completed the molecular characterization of a missense mutation (p.Leu205Pro). The mutant p.Pro205 protein was detected in the cell lysates in a similar amount to WT AT, based on this the protein synthesis was not affected. In contrast, only a small amount of mutant protein was expressed into the supernatant of the cells, suggesting a secretion defect. The specific activity of the Pro205 protein was also decreased comparing to WT AT. In summary, we concluded, that the mutation led to a complex defect.
- The 5 patients with Osler-Rendu-Weber disease are from the close geographic region, and the carriership of the same mutation (*ACVRL1* c.625+1 G>C) suggested a founder mutation. This hypothesis was confirmed by genotyping 8 polymorphic genetic markers and by genealogical analysis.

7. SUMMARY

Antithrombin (AT) is a key regulator of the coagulation. AT deficiency is a rare but major risk factor in venous thrombosis. It can be classified as type I (quantitative) and type II (qualitative) deficiency. The molecular genetic background of AT deficiency is heterogeneous, more than 310 mutations have been described in the gene encoding AT (*SERPINC1*).

Thirty-one *SERPINC1* mutations including 11 novel ones with a high mutation detection rate (98%) were diagnosed in 156 non-related AT deficient patients (n=246 with family members). Heparin binding site deficiency (type II.HBS) was the most frequent (75.6%) including AT Budapest3 (AT Bp3, 86,4% of II.HBS), AT Padua I (9%) and AT Basel (4%). The presence of a founder effect concerning AT Bp3 was confirmed by the investigation of 12 polymorphic markers. Type II.HBS deficiencies behave differently in clinical and laboratory phenotypes from each other and from other AT deficiencies. Arterial thrombosis and pregnancy complications were the most frequent in AT Basel and AT Padua I mutations, respectively. Median age at the time of thrombosis was the lowest in AT Bp3 homozygotes. By investigating three different functional anti-FXa based assays, it was suggested that beside the source of the enzyme other assay conditions are also crucial for the sensitivity of the assay. The functional assay with high heparin concentration and pH 7.4 as assay conditions had low (44%) sensitivity for AT Bp3 and it was absolutely insensitive for AT Basel and Padua I. Heparin concentration and pH seem to be the key factors influencing the sensitivity of AT functional assays to II.HBS. The novel pathogenic p.Leu205Pro mutation showed a complex phenotype in the in vitro expression studies, where not only the secretion but also the specific activity of the mutant AT was altered.

The Osler-Rendu-Weber disease (or hereditary hemorrhagic telangiectasia; HHT) is a rare autosomal dominant vascular abnormality characterized by mucocutaneous telangiectases and visceral arteriovenous malformations. The clinical diagnosis of the disease is based on the four Curaçao criteria.

A novel splicing mutation (*ACVRL1* c.625+1 G>C) was identified in 5 families living in North-East of Hungary. Haplotype analysis ascertained the possibility of a founder effect in the case of *ACVRL1* c.625+1 G>C. Mutation carriers, their spouses and 50 healthy people were genotyped for 8 polymorphic markers within and outside of *ACVRL1* gene on chromosome 12. Founder effect was also demonstrated by genealogical methods.

8. LIST OF PUBLICATIONS



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Candidate: Réka Gindele
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List of publications related to the dissertation

1. Selmeczi, A.*, Gindele, R.*, Ilonczai, P., Fekete, A., Komáromi, I., Schlamadinger, Á., Rázsó, K., Kovács, K. B., Bárdos, H., Ádány, R., Muszbek, L., Bereczky, Z., Boda, Z., Oláh, Z.:
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Thromb. Res. 158, 1-7, 2017.
DOI: <http://dx.doi.org/10.1016/j.thromres.2017.07.023>

*These authors equally contributed to the work.
IF: 2.65 (2016)
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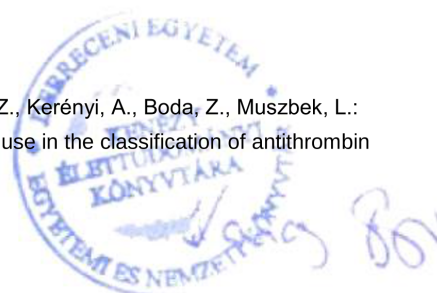




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