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

Characterization of *SERPINC1* mutations causing antithrombin deficiency

by

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Head of the **Examination Committee**:	Prof. József Balla, PhD, DSc, MHAS
Members of the Examination Committee:	Imre Bodó, MD, PhD
	Katalin Gombos, MD, PhD

The Examination takes place at the Library of Division of Clinical Laboratory Science, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, at 11:00 a.m., 3rd of December, 2024.

Head of the **Defense Committee**:	Prof. József Balla, PhD, DSc, MHAS
Reviewers:	Hussain Alizadeh, MD, PhD
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Members of the Defense Committee:	Imre Bodó, MD, PhD
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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 p.m., 3rd of December, 2024.

1. INTRODUCTION

Hemostasis allows the blood to maintain its liquid state inside the blood vessel, but to solidify when leaving it, thus prevent an injury of the vessel wall to cause bleeding. Disruption of the gentle balance of hemostasis has serious consequences, bleeding and thrombosis. A change in three factors can lead to thrombus formation, which was first described by Rudolf Virchow, a German pathologist in the 19th century. The three factors are changes in blood flow (stasis), vessel wall (vessel wall disorder) or blood coagulability (hypercoagulability). Congenital factors leading to hypercoagulability include antithrombin (AT), protein C and S (PC, PS) deficiencies, activated protein C resistance (APC resistance, its main cause is the FV Leiden mutation), the prothrombin gene (F2) 20210A allele and certain types of dysfibrinogenemias. These conditions cause an increased risk of venous thromboembolism (VTE). The risk of VTE is also increased by hyperhomocysteinemia and antiphospholipid syndrome, in which the presence of lupus anticoagulant and anticardiolipin antibodies can be detected in the circulation. Antithrombin belongs to the superfamily of serine protease inhibitors (SERPINs). It is the major plasma inhibitor of thrombin and activated factor X (FXa); however, it also inactivates FIXa, FXIa, FXIIa and FVIIa. In the presence of heparin or heparan sulfate proteoglycans, the rate of inhibition is accelerated.

Antithrombin deficiency (ATD) is the most severe inherited thrombophilia according to our present knowledge, and it may be inherited or acquired. A decrease in functional antithrombin in plasma results in an increased risk of thromboembolism. 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.

Two major forms of ATD are recognized: type I and II. Type I (quantitative) deficiency, which has been identified only in the heterozygous state, is characterized by a reduction in AT concentration to ~50% of normal. In contrast, type II (qualitative) deficiencies are characterized by the presence of a dysfunctional protein in the plasma. The defect may affect the reactive site (II RS) or the heparin binding site (II HBS), or it can cause a pleiotropic effect (II PE). According to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk; last accessed at 11 November 2023), n = 529 different mutations have been reported thus far in *SERPINC1*. In most cases, missense/nonsense mutations have been registered (n > 231), but splicing variants, small deletions and insertions have also been presented. Detailed clinical and molecular characterizations of the identified mutations provide information to understand

the pathophysiology of the disease and help in the classification of novel mutations according to pathogenicity.

The first part of the present study was to investigate the molecular background of the differences among type IIHBS AT variants (ATBp3, AT Basel, AT Padua) focusing on their heparin-binding affinity. AT Basel was associated with arterial thrombosis in the early report of Brennan, and the mutant AT showed an abnormal peak in heparin-Sepharose affinity chromatography. In a monoclonal antibody-based heparin-binding affinity assay AT Basel demonstrated 40-fold reduction in heparin-binding affinity comparing it to normal AT. AT Padua showed 30-fold decrease in heparin affinity in another study using other method for investigation. The ATBp3 variant showed reduced heparin affinity by heparin-Sepharose chromatography, and the antiproteinase activity was decreased as compared to normal AT in the presence of unfractionated heparin (UFH) or the AT-binding pentasaccharide. According to the results of an elegant study of Martinez-Martinez et al. plasma samples of ATBp3 homozygous patients showed an increased fraction of AT with low heparin affinity. The major finding of that study was the demonstration of the compensatory role of β-AT in the type IIHBS mutants ATBp3 and AT Basel. They did not investigate AT Padua, however in case of an Arg to Cys mutation in the same position the recombinant β-isoform could compensate for the strong effects of this mutation in the interaction with heparin. Direct comparison of the heparin-binding features of ATBp3, AT Basel and AT Padua by using uniform methodology for all mutants, however, has not performed, as yet.

We identified nine novel AT gene mutations in patients with thrombosis. In the second part of the thesis, we investigated the genotype–phenotype correlations and the molecular characteristics of these mutations. Extensive in vitro characterization of AT mutations has been demonstrated in only a few studies.

1.1. The SERPINC1 gene and the structure of antithrombin

The antithrombin gene (*SERPINC1*) is mapped to chromosome 1q23.1–23.9 and comprises seven exons and six introns spanning a total of 13.5 kb of genomic DNA. The heparin-binding part of AT is encoded by the second and third exons, and the reactive site located at the C-terminal part is encoded by the seventh exon. Nine full and one partial Alu repeats were identified for introns 1, 2, 4, 5 and 6. The signal peptide of 32 amino acids directs the translocation of the protein to the endoplasmic reticulum, where it is folded and subjected to two post-translational modifications: formation of 3 intramolecular disulfide bonds and N-glycosylation at Asn128, 167, 187 and 224. Mature antithrombin (AT) is a 58 kDa plasma

glycoprotein that consists of 432 amino acid residues with three intramolecular disulfide bridges at Cys40-160, Cys53-127 and Cys279-462. 2 glycoforms are present in the circulation, 90-95% is the α-glycoform and less than 10% is the β-glycoform. Asn167 residue of β-AT lacks glycosylation, as a result of which it binds more strongly to heparin and similar molecules. For example, heparan sulfate, which is present on the surface of endothelial cells and in most cells. AT is a serine protease inhibitor with a characteristic secondary and tertiary structure, which contains 9 α helices (A – I) and 3 β folds (A – C). The reactive center loop (RCL) is located at the top of the molecule, this part contains complementary sequences to the active site of serine proteases. SERPINs have significant structural flexibility, changes in their structure contribute to the inhibition of serine proteases. In the uncleaved state, two main conformational states are distinguished, the native and the latent form. In the case of the native form, the reactive central loop is located on the surface of the AT molecule, in the case of the latent configuration, the RCL is inserted between the β-folds. The latent form is thermodynamically more stable, yet the native form trapped at a higher energy level is present in circulation. Binding of pentasaccharide or heparin containing a pentasaccharide unit causes conformational changes in and near the RCL. The interaction between the pentasaccharide unit and AT takes place in two steps, an initial weakly bound intermediate is transformed into a strongly bound form.

1.2. The role of antithrombin in blood coagulation and other functions

Physiological regulation of blood coagulation requires temporal and spatial limitation of thrombin generation and action. Hereditary lack or dysfunction of physiological inhibitors may be associated with an increased risk of thrombosis. The blood coagulation inhibitory system consists of various protease inhibitors (antithrombin, α1-antitrypsin, α2-macroglobulin, Tissue factor pathway inhibitor and heparin cofactor II), which are found in the blood circulation and make up more than 10% of blood plasma proteins. Their mechanism can be two types: in case of one mechanism, the enzyme binds to the inhibitor, but the active center of the enzyme remains functional (for example, α2-macroglobulin), during the other mechanism, a covalent bond is formed between the active center of the enzyme and the inhibitor. This is how AT, α1-antitrypsin and heparin cofactor II work. All three inhibitors can be considered as substrates of coagulation serine proteases. The inhibited enzyme creates an equimolar complex with the inhibitor, so it not be able to perform any further function. The inhibitory effect of AT is most pronounced towards thrombin and active factor X (FXa), but it also inhibits other serine protease coagulation factors. The AT molecule binds with high

affinity to heparin or heparan sulfate proteoglycans. This binding greatly enhances the inhibitory effect of AT on activated coagulation factors (e.g. thrombin, FXa).

During the inhibition of thrombin, both AT and thrombin bind to heparin with high affinity, then a covalent bond is formed between the active center of that enzyme and the inhibitor (thrombin is inhibited). This interaction also occurs without heparin, but with a much higher reaction rate in the presence of heparin. Since the already formed thrombin-antithrombin complex has a low affinity for heparin, heparin is released and can reenter the next reaction. The mechanism of FXa inhibition is slightly different from the thrombin inhibition mechanism, here it is not necessary for FXa to bind to heparin, so its inhibition occurs effectively even in the presence of shorter saccharide units (the presence of LMWH or pentasaccharide is sufficient). Inhibition of thrombin requires the presence of heparin consisting of at least 18 saccharide units, as it must bind to heparin.

The primary function of AT is therefore to prevent the formation of thrombin-mediated fibrin clots and to suppress the generation of thrombin created by FXa, and the so-called inhibition of other activated coagulation factors involved in intrinsic and extrinsic pathways (FIXa, FXIa, FXIIa, FVIIa-tissue factor complex).

Heparin is one of the most frequently used drugs in the prevention and treatment of thromboembolic diseases for several decades due to its ability to significantly accelerate the effect of AT. Unfractionated heparin (e.g. Heparibene) consists of mucopolysaccharide-polysulfonic acid ester, glucosamine-N-sulfuric acid and glucuronic acid-sulfuric acid esters, which are connected to each other by glycosidic means (polymer). Due to its strong negative charge, it forms a complex with certain proteins (e.g. AT). The group of low molecular weight heparins (LMWH) includes enoxaparin (Clexane), which is a low molecular weight heparin of approximately 4500 daltons. The active substance of the preparation is a sodium salt, which has strong anti-FXa activity and weak anti-FIIa activity. The smallest unit of heparin action is fondaparinux sodium (e.g. Arixtra), which is a synthetic pentasaccharide. A selective inhibitor of activated FX, this effect is mediated by AT. It does not inactivate thrombin. (Source: Heparibene, Clexane, fondaparinux instructions for use).

In addition to the anticoagulant function of the AT protein, several other functions are also known in the human body. AT can bind to specific heparan sulfate proteoglycan receptors (syndecan 4) on the surface of endothelial cells through the D-helix, similar to the binding of heparin given for therapeutic purposes. This signaling activity of AT induces prostacyclin synthesis in endothelial cells, thereby inhibiting the nuclear factor-kappaB pathway and the synthesis of proinflammatory cytokines and adhesion proteins. AT also has a strong anti-

angiogenic activity, however, this is only characteristic of its cleaved and/or latent conformation (RCL enters between the β-folds). Both the cleaved and latent forms have very low heparin affinity. These low-affinity AT forms do not show anti-inflammatory functions either, but have strong proapoptotic signaling activity on endothelial cells. It has been described that both the anti-inflammatory and anti-angiogenic/proapoptotic properties of AT are realized through the interaction through the D-helix. One study attributed the antibacterial effect of AT to the binding of β-AT to Gram-negative bacteria, including E. coli, and to the purified cell wall component of the bacteria. The binding of β-AT to the bacterial cell wall can increase the phagocytic activity of macrophages and thereby eliminate the bacteria from the body. The antibacterial effect is also assumed to play a role in the D-helix.

The anti-inflammatory signaling function of AT was investigated in endothelial cells and the necessity of the D helix-dependent interaction with the syndecan 4 receptor was demonstrated. Signaling was also investigated in monocytes and it was shown that in their case the syndecan 4 receptor is specific for β-AT.

All these support the essential role of AT in the body, which is realized not only through its antithrombotic effect, but also through its anti-inflammatory and anti-angiogenic/proapoptotic effects.

1.3. Antithrombin deficiency

Antithrombin deficiency (ATD) can be acquired or inherited. To briefly mention a few reasons for the acquired form: it can develop as a result of reduced AT synthesis due to liver failure, in nephrotic syndrome due to increased AT loss, in sepsis or acute disseminated intravascular coagulation due to increased AT use and inactivation. Drug-induced cases have also been described. For example, L-asparaginase therapy, long-term, high-dose heparin therapy. Inherited ATD can be divided into two groups, type I (quantitative) and type II (qualitative). It is mostly inherited in an autosomal dominant manner and affects both men and women. Inherited ATD usually occurs in a heterozygous form, as homozygous cases are rarely fatal in utero. The quantitative defect is characterized by a proportional decrease in AT antigen level and activity. Type II can be further divided into 3 subgroups according to the location of the defect, such as affecting the reactive site (IIRS), affecting the heparin binding site (IIHBS) or pleiotropic (IIPE). In addition to the AT antigen level usually within the reference range for a qualitative defect, a decrease in the activity of the AT molecule can be seen, which is caused by the defective AT protein. Homozygous patients can also occur in the less severe IIHBS subtype. The prevalence of ATD is estimated to be approximately 1:2000.

However, this number is higher among patients with thrombosis, in their case it is presumably between 1:20 and 1:200, but we do not have exact data on this, mainly due to population and methodological differences and the rarity of the disease.

Inherited AT deficient individuals have an increased risk of thromboembolic events, primarily venous, but arterial thrombosis is also described. It is now known (partially from previous results of our own research group) that ATD subgroups do not necessarily show the same clinical phenotype. Furthermore, even within the same subgroup, there may be phenotypic differences that are characteristic of certain mutations. According to a Japanese study, type I was more common and more severe than type II ATD. Loss-of-function mutations in type I ATD were associated with more severe symptoms (younger age or recurrent VTE) compared with other mutation types. In the French study with the largest number of patients, the risk of venous thromboembolism (VTE) was lower in type IIHBS compared to type I. Type IIHBS ATD is more frequently associated with arterial thromboembolism (ATE) than type I and other type II deficiencies. A smaller study from Denmark observed that type I and type IIRS were more severe (causing VTE more often) than IIHBS Basel. Interestingly, the clinical symptoms of IIHBS AT deficiencies show significant heterogeneity, which we observed in our clinical study involving 246 AT deficiency patients, as 75% of the patients were IIHBS type patients with varied clinical phenotypes. In a study from Finland, where the prevalence of AT Basel (p.Pro73Leu) is relatively high, this type was associated with a higher risk of thrombosis, and ATE and pregnancy complications were also common. Another study investigated 82 IIHBS ATD patients with 6 different mutations, including AT Basel and AT Padua (p.Arg79His) mutations. They found that in their study group, VTE was common and ATE was more often associated with AT Basel and Padua mutations compared to type I. Since AT Budapest 3 (p.Leu131Phe) is the founding mutation in Hungary, it was possible to examine a large number of such patients and compare their clinical phenotype with other IIHBS types. While AT Budapest 3 (ATBp3) homozygosity represents the most severe thrombophilia (even more severe than type I) and is also associated with VTE, AT Basel is more associated with ATE and AT Padua shows more pregnancy complications. ATBp3 heterozygous status has been described in selected ATE patients. We must take into account, however, that in the case of the same inherited ATD, the frequency of occurrence of associated acquired risk factors predisposing to thrombosis may differ significantly in the case of different study populations.

Laboratory diagnosis of ATD is based on a functional test where AT is active for factor X (FXa) or thrombin (FIIa) is measured in the presence of heparin. It has been observed

that currently available functional tests detect certain ATD subgroups with different sensitivities, making laboratory diagnosis difficult. The AT Cambridge II (p.Ala416Ser) type IIRS mutation is relatively common in the AT deficient population of the United Kingdom, for which FXa-based tests have been shown to be insufficiently sensitive. In contrast, it can generally be said that FXa-based tests in the presence of heparin (hc-anti-FXa) are much more sensitive to IIHBS ATD compared to FIIa-based tests. By performing the test without heparin, the result of progressive AT activity (p-anti-FXa) is obtained. Progressive AT activity in proportion to heparin cofactor AT activity is definitely increased in IIHBS ATD. It has been shown that hc-anti-FXa AT activity among IIHBS patients varies depending on the specific mutation. Since different commercially available tests detected the ATBp3 homozygous, ATBp3 heterozygous, AT Basel and AT Padua heterozygous mutants with different sensitivities, it can be assumed that not only the enzyme (FIIa or FXa), but also other reaction conditions, such as heparin concentration or ionic strength was responsible for the heterogeneous results. High heparin concentration assays showed lower sensitivity in AT Basel and AT Padua cases than in ATBp3, thus pointing to differences in AT-heparin binding strength and AT activation between these mutants.

During the laboratory diagnosis of ATD, if a reduced heparin cofactor AT activity is obtained (hc-anti-FXa), the next step is an AT antigen determination, on the basis of which the patient can be classified as type I or II. A normal AT antigen level indicates type II and in this case progressive AT activity is performed. Low progressive AT activity is characteristic of type IIRS and IIPE, while normal progressive activity suggests the presence of IIHBS mutation. In case of suspected IIHBS, the ATBp3 mutation is first detected in our laboratory using a specific genotyping method developed for ATBp3 (this is because ATBp3 is extremely common in our country due to the founder effect). If this turns out to be negative, specific genotyping is performed for some more common IIHBS mutations. If these do not reveal the causal mutation either, the entire SERPINC1 gene is sequenced. If new mutations are detected, they can be analyzed biochemically and in silico, and their pathogenicity can be determined. After creating the mutation with mutagenizing primers, we have the possibility of transient and stable transfection of HEK-293 cells and the expression of the mutant AT protein. The stable transfection of the cells is optimal for the production of a larger amount of recombinant protein, which is used in the functional tests after purification.

The pathogenicity of new mutations can only be established on the basis of direct and indirect evidence, guidelines help in the classification of mutations.

New mutations - based on different genetic/genomic recommendations - can be classified into the following groups: benign, probably benign, variant of unknown significance, probably pathogenic and pathogenic. The effect of a mutation on a patient's phenotype can be diverse. A given mutation can be benign or pathogenic. The pathogenicity of a specific mutation can be strongly supported (e.g. the consequence of the mutation is a null variant) or less strong (e.g. a mutation that changes the length of the protein).

2. AIMS OF THE STUDY

We planned a comparative biochemical and in silico study of three known mutations (ATBp3, AT Basel, AT Padua) affecting the AT heparin-binding site. These mutations frequent in ATD patients diagnosed at the Division of Clinical Laboratory Science, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen. We planned to characterize the mechanism of heparin binding in detail.

We planned the biochemical and in silico characterization of nine new SERPINC1 mutations (p.Arg14Lys, p.Cys32Tyr, p.Arg78Gly, p.Met121Arg, p.Leu245Pro, p.Leu270Argfs*14, p.Asn450Ile, p.Gly456delins_Ala_Thr and p.Pro461Thr) found in ATD patients diagnosed at the Division of Clinical Laboratory Science, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen. We aimed to prove their pathogenicity.

3. MATERIALS AND METHODS

3.1. Clinical and Routine Laboratory Data of Antithrombin Deficient Patients

Between January 2007 and December 2021, a total of 478 non-related AT deficient patients were diagnosed at our center. Blood samples were collected into 0.109 M citrated vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ) and plasma samples were stored at -80°C until analysis. Inherited thrombophilia (protein C and S deficiencies, APC resistance, dysfibrinogenemia) was investigated by routine laboratory methods with a BCS-XP coagulometer (Siemens, Mar-burg, Germany). 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 detected with a Siemens BCS-XP coagulometer. AT antigen was measured by immunonephelometry (Siemens, N Antiserum to Human Antithrombin III, reference interval 0.19–0.31 g/L). Clinical data were collected retrospectively from the patients. Information on the type and date of the first thrombotic

event was obtained from medical records. Ethical approval for the study was obtained from the National Ethical Council (3166/2012/HER).

3.2. Mutation Analysis of SERPINC1 Gene in Antithrombin Deficient Patients

Genomic DNA was isolated from peripheral whole blood using QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany). Sanger sequencing was performed to identify mutations in the exons, the flanking intronic regions and in the promoter of SERPINC1 gene using an ABI3130 Genetic Analyzer and Sequencing Analysis 5.4 software (Thermo Fisher Scientific, Carlsbad, CA, USA). Multiplex ligation-dependent probe amplification (MLPA) was performed using SALSA MLPA KIT P227 (MRC-Holland, Amsterdam, the Netherlands) using an ABI3130 Genetic Analyzer if Sanger sequencing was negative. The MLPA products were analyzed with GeneMapper Software 4.1 (Thermo Fisher Scientific).

3.3. Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed according to Sas et al. The first electrophoresis was performed at 150 V for 60 minutes in 1% agarose containing 16.3 U/mL of unfractionated sodium heparin. The second dimension electrophoresis was performed at 100 V for 180 minutes in 1% agarose containing 1% rabbit anti-human AT anti-serum (Sigma, Saint Louis, MO, USA).

3.4. NanoDSF

NanoDSF is a differential scanning fluorimetry method used for accurate analysis of protein folding and stability. NanoDSF measures the intrinsic tryptophan or tyrosine fluorescence for the analysis. During unfolding of proteins tryptophan becomes hydrated and its fluorescence intensity maximum is shifted from 330 nm to 350 nm. The thermal stability of a protein can be described by the thermal unfolding transition midpoint (Tm) at which half of the protein population is unfolded. It could also be characterized by the onset temperature (Tonset) of the denaturation. Tm can be determined from the inflection point of the denaturation curve, which is the ratio of the tryptophan fluorescence at 330 and 350 nm plotted against the temperature or from the maximum of its first derivative. The dual wavelength system of the Prometheus NT.48 (NanoTemper Technologies GmbH, Munich, Germany) was used to characterize the thermal unfolding processes of wild type and Bu-dapest3 mutant form of AT protein. The samples were loaded into standard glass capillaries at a concentration of 8 μM in triplicate. The sample volume was about 10 μL per capillary. During the analysis the samples were

heated from 20 °C to 95 °C at a ramp rate of 1 °C/min. Tm and Tonset values were determined using PR.ThermControl v2.1.2 software using first derivative analysis of 350 nm/330 nm fluorescence ratio plotted against the temperature.

3.5. In Vitro Expression of Wild Type and Mutant Antithrombins

The cDNA clone ORF-NM_000488_pcDNA3.1(+) wild-type AT (WT) was purchased from ImaGenes GmbH (Berlin, Germany). The twelve mutant plasmids (p.Leu131Phe, p.Pro73Leu, p.Arg79His, p.Arg14Lys, p.Cys32Tyr, p.Arg78Gly, p.Met121Arg, p.Leu245Pro, p.Leu270Argfs*14, p.Asn450Ile, p.Gly456delins_Ala_Thr and p.Pro461Thr) were created by us using the QuickChange Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA, USA) kit according to the manufacturer's instructions. Primers for the mutagenesis (BioScience, Integrated DNA Technologies, BVBA, München, Germany):

p.Leu131Phe (ATBp3):

primer 1: 5' – GCCTGTAATGACACCTTCCAGCAACTGATG – 3'

primer2: 5' – CATCAGTTGCTGGAAGGTGTCATTACAGGC – 3'

p.Pro73Leu (AT Basel):

primer 1: 5' – GGCTCAGAACAGAAGATCCTGGAGGCCACCAAC – 3'

primer 2: 5' – GTTGGTGGCCTCCAGGATCTTCTGTTCTGAGCC – 3'

p.Arg79His (AT Padua):

primer 1: 5' – GCCACCAACCGGCATGTCTGGGAACTGTC – 3'

primer 2: 5' – GACAGTTCCCAGACATGCCGGTTGGTGGC – 3'

p.Arg14Lys:

primer 1: 5' – TAACCTCTGGAAAAAAGAAGGTTTATCTTTTGTCCT – 3'

primer 2: 5' – AGGACAAAAGATAAACCTTCTTTTTTCCAGAGGTTA – 3'

p.Cys32Tyr:

primer 1: 5' – GGACTGCGTGACCTATCACGGGAGCCCTGTGGAC – 3'

primer 2: 5' – GTCCACAGGGCTCCCGTGATAGGTCACGCAGTCC – 3'

p.Arg78Gly:

primer 1: 5' – AGGCCACCAACGGGCGTGTCTGGGAACTG – 3'

primer 2: 5' – CAGTTCCCAGACACGCCCGTTGGTGGCCT – 3'

p.Met121Arg:

primer 1: 5' – CGGCTTTTGCTAGGACCAAGCTGGGTGCC – 3'

primer 2: 5' – GGCACCCAGCTTGGTCCTAGCAAAAGCCG – 3'

p.Leu245Pro:

primer 1: 5’ – CAATGAGCTCACTGTTCCGGTGCTGGTTAACACC – 3’

primer 2: 5’ – GGTGTTAACCAGCACCGGAACAGTGAGCTCATTG – 3’

p.Leu270Argfs*14:

primer 1: 5’ – CACAAGGAAGGAACGTTCTACAAGGCTGAT – 3’

primer 2: 5’ – ATCAGCCTTGTAGAACGTTCCTTCCTTGTG – 3’

p.Asn450Ile:

primer 1: 5’ – GAGAAGTTCCTCTGATCACTATTATCTTCATGGGC – 3’

primer 2: 5’ – GCCCATGAAGATAATAGTGATCAGAGGAACTTCTC – 3’

p.Gly456delins_Ala_Thr:

primer 1: 5’ - CTATTATCTTCATGGCTACAAGAGTAGCCAACCC – 3’

primer 2: 5’ - GGGTTGGCTACTCTTGTAGCCATGAAGATAATAG – 3’

p.Pro461Thr:

primer 1: 5’ – GGGCAGAGTAGCCAACCCTTGTGTTAAGTAA – 3’

primer 2: 5’ – TTACTTAACACAAGGGTTGGCTACTCTGCCC – 3’

HEK293 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, High glucose, Biosera) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine and 25 μg/mL gentamicin antibiotic (Chinoin, Budapest, Hungary) at 37 °C and 5% CO2 in a humidified incubator. For all experiments, cells were grown to 60–80% confluency and were subjected to no more than 20 cell passages. Cells were subcultured every 3 days using a standard trypsinization procedure. Transient transfection of the WT and the nine mutant AT plasmids was performed using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) and co-transfection of the LacZ gene was also performed with pCMV Sport β-GAL plasmid (Invitrogen, Carlsbad, CA, USA). After 48 h of incubation, conditioned media were collected, and the cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and a protease inhibitor cocktail (Roche). A FluoReporterlacZ/Galactosidase Quantitation Kit (Molecular Probes, Life Technologies) was used to investigate the transfection efficiency, and the results were corrected accordingly.

3.6. Stable Transfection of Wild-Type and Mutant Antithrombins

Stable transfection of HEK-293 cells with huSERPINC1_pcDNA3.1(+) wild_type and huSERPINC1_pcDNA3.1(+) _ATBp3, huSERPINC1_pcDNA3.1(+) _AT Basel, huSERPINC1_pcDNA3.1(+) _AT Padua, huSERPINC1_pcDNA3.1(+)_p.Arg14Lys _type, _ p.Cys32Tyr_type, _ p.Arg78Gly_type, _ p.Met121Arg_type, _ p.Leu245Pro_type and _

p.Pro461Thr_type plasmids, respectively, was performed as follows. The first step was the generation of a kill curve to determine the optimal selection antibiotic concentration for selecting stable cell colonies. A kill curve is a dose–response experiment where the HEK293 cells are subjected to increasing amounts of geneticin (0–1000 µg/mL) to determine the minimum concentration that kills all the cells within 10 days. We used Geneticin® Selective Antibiotic (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA) as a selective agent in our stable transfection experiments. Resistance to geneticin is conferred by the neomycin resistance gene which is in the pcDNA3.1(+). We found a 400 µg/mL geneticin concentration optimal. The second step was the transfection of the wild type and the six mutant antithrombin plasmids. Cells were plated in a 24-well dish (reaching about 70–80% confluence) and transfected the following day. Transfection was performed by addition of 2.0 µg plasmid DNA encoding for the wild-type and the mutant AT in reduced serum Opti-MEM medium (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA) with a Lipofectamine® 3000 Transfection Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The third step was the selection for transfected cells. After 24 h, 400 µg/mL geneticin was added to select the resistant colonies. The selection medium was replaced every three days. We cultured the cells in selective medium for ten days. Most of the cells that had not integrated the transfected plasmid died, while the cells that had undergone plasmid integration survived. The surviving cells were allowed to expand; when the cells in the T75 culture flask reached high confluence, we froze them as a polyclonal line.

3.7. Preparation of Antithrombin from in vitro Expressed Recombinant Antithrombins and from Normal and ATBp3 Homozygous Plasma by Affinity Chromatography

AT proteins (WT, ATBp3, AT Basel and AT Padua) were purified from the concentrated culture media of transfected HEK-293 cells by affinity-chromatography using Goat anti-human Antithrombin IgG (Affinity Biologicals, Ancaster, Canada) that was covalently coupled to Sepharose 4B gel. The concentration of purified AT proteins was determined by immunonephelometry (Siemens). Normal and ATBp3 homozygous plasma samples were also purified by this protocol.

3.8. Surface Plasmon Resonance

Surface plasmon resonance (SPR) assays were performed on a Biacore 3000 instrument (GE Healthcare, Uppsala, Sweden). For assaying the binding characteristics of different AT mutants to heparin, heparin SPR sensorchip (Heparin Approx. 50 nm hydrogel chip, XanTec

bioanalytics GmbH, Dusseldorf, Germany) was used. One of the advantages of the device used is that it can divide the sensor chip into 4 cells. We were, therefore, able to test all the four recombinant (WT and 3 mutant) AT proteins on the same chip. A total of 90 µL of each AT mutants, diluted in running buffer (HEPES 10 mM, NaCl 150 mM, EDTA 3 mM, surfactant 0.005% [v/v], pH 8.4), was injected into the microflow cell in 6 different concentrations (50, 100, 150, 300, 500, 750 nM). The flow rate was 10 µL/min. Between two measurements chips were regenerated with 30 µL regeneration buffer (10 mM glycin-HCl, pH 2.5, GE healthcare, Uppsala, Sweden). Langmuir 1:1 binding model was used for curve fitting. From the sensorgrams, the association and dissociation rate constants (k_a and k_d), and the equilibrium association and dissociation constants (K_A and K_D) were calculated by the BIAevaluation software (GE Healthcare, Uppsala, Sweden). Optimal pH of the reaction was previously determined (pH 8.4) and the reaction proceeded at this pH.

3.9. AT Antigen and Activity Measurements in the Conditioned Media Containing Each Recombinant AT Protein

Aliquots of the conditioned media and cell lysates were used for AT antigen determination by ELISA (Abcam Human-Antithrombin-III-ELISA-Kit, Abcam, Cambridge, UK). AT activity from the conditioned media of the transfected cells was detected through an amidolytic assay in a microtiter plate using LX Antithrombin Hc + P, FXa reagent (Labexpert Ltd., Debrecen, Hungary) with minor modifications. Briefly, this assay comprised bovine FXa as a substrate and BIOPHEN CS-11(32) [Suc-Ile-Gly-(γ Pip)Gly-Arg-pNA, HCl] as a chromogenic substrate (HYPHEN Biomed., Neuville, France). We used a final dilution of 1:2 of conditioned media of transfected HEK293 cells in a Tris-HCl buffer (pH 8.4) containing heparin (heparin cofactor activity). We performed the assay with the same conditions in the absence of heparin (progressive activity).

3.10. N-Glycosidase F Digestion

N-Glycosidase F cleaves asparagine-bound N-glycans from glycoproteins. The AT variants (p.Met121Arg, p.Leu245Pro, p.Asn450Ile and p.Gly456delins_Ala_Thr) were treated with N-Glycosidase F (Roche Diagnostics GmbH, Mannheim, Germany). Before PNGase F digestion, the samples were pretreated with 150mM Na-phosphate at 95 °C for 5 min. Then, we added 6 µL 100 U/mL PNGase F enzyme and incubated the samples at 37 °C for 15 h. Samples were resolved on 10% SDS-PAGE gels and then transferred to nitro-cellulose membranes. Through Western blot analysis, the AT specific immunoreactive bands were

visualized with enhanced chemiluminescence (ECL) detection (Thermo Fisher Scientific, Carlsbad, CA, USA).

3.11. Western Blot Analysis

Conditioned media were collected; then, the cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and a protease inhibitor cocktail (Roche) and centrifuged at 12,000× g for 15 min at 4 °C. The total protein concentrations of the extracts were measured with a Pierce BCA pro-tein assay kit (Thermo Fisher Scientific). Equal amounts of protein were loaded onto 10% SDS-PAGE gels and then transferred to nitrocellulose membranes. After the membranes were blocked at room temperature for 1 h in 5% nonfat dry milk in TBS-T buffer, they were incubated overnight with primary antibodies at 4 °C. AT was detected with a goat anti-human AT antibody (Affinity Biologicals, Ancaster, Canada); beta tubulin polyclonal antibody (Invitrogen) was used as a loading control. The primary antibodies were diluted 1:10,000 in blocking solution. The membranes were washed three times for 7 min with TBS-T buffer and incubated for 45 min at room temperature with the appropriate secondary antibody. Horseradish peroxidase-conjugated anti-goat IgG (Abcam) and anti-rabbit IgG (GE Healthcare) were diluted 1:10,000 in blocking solution. Immunoreactive bands were visualized with ECL following the manufacturer's instructions (Thermo Fisher Scientific). Chemiluminescent imaging was performed with a C300 Azure Imaging system (Azure Biosystems, Dublin, CA, USA).

3.12. Real-Time Quantitative PCR Analysis

We performed real-time quantitative RT-PCR to investigate the mRNA expression levels of the wild-type form and the nine mutant AT forms in transient transfection. Total RNA was isolated from transiently transfected HEK293 cells with QIAamp RNA Blood Mini Kit (QIAGEN). We used a RapidOut DNA Removal Kit (Thermo Scientific) to remove the genomic DNA, and after reverse transcription (qPCRBIO cDNA Synthesis Kit), real-time quantitative PCR was performed using the following primers: AT RNA forward primer 5′ – GCTAAACCCCAACAGGGTGA-3′; AT RNA reverse primer 5′ – TTACTTAACACAAGGGTTGGCTAC-3′; OAZ RNA forward primer 5′ – CACCATGCCGCTCCTAAG-3′; OAZ RNA reverse primer 5′ – GAGGGAGACCCTGGAACTCT-3′; β-GAL RNA forward primer 5′ – GCGTACATCGGGCAAATAAT-3′; β-GAL RNA reverse primer 5′ – TAATCACGACGCGCTGTATC-3′.

3.13. In silico methods

3.13.1. Investigation of IIHBS ATD mutations (ATBp3, AT Basel, AT Padua) with in silico methods

To investigate the effects of the mutations on the AT heparin pentasaccharide binding, as well as the stability of the protein, we have built two types of model systems. First, the 1T1F X-ray diffraction structure was used for the system not containing the pentasaccharide, representing the "non-activated" state. Second, the pentasaccharide bound, activated AT was modelled starting from the X-ray structure 1NQ9. We have constructed models corresponding to both activation states for WT AT, as well as the three mutants, ATBp3, AT Basel and AT Padua. The latter variant was simulated with both neutral and protonated His79 (His47 in the mature protein).

Similarly to our previous paper regarding the AT – pentasaccharide binding, we have chosen the CHARMM36m force field for the AT protein and the CHARMM carbohydrate FF for the pentasaccharide. Like β-AT, the protein was glycosylated on three Asn residues: Asn-96, -155 and -192 in the mature protein. Due to their large size, the oligosaccharide chains were truncated in the same way as in the article mentioned previously. We used the CHARMM-GUI web server to generate the topology files for all three systems.

Thus, the appropriate sampling of such conformational transitions often requires advanced sampling techniques. The Gaussian Accelerated Moleculer Dynamics (GAMD) method was chosen because it offers significantly enhanced sampling while it does not depend on pre-defined reaction coordinates.

The AMBER 16 pmemd.cuda software was used for the molecular dynamics (MD) and GAMD simulations, (ambermd.org). All model systems were subjected first to two consecutive energy minimizations, 2000 steps each. The first minimization included 500 steps using the steepest descent and 1500 steps of conjugate gradient method, with the protein and the ligand position restrained. In the following 2000 steps, no residues were restrained and the conjugate gradient method was applied. The system was then heated from 0 K to 310 K in a 2 ns MD simulation, with all non-solvent and non-ion atoms restrained. The heating was followed by 2 ns of pressure equilibration.

A dual-boost scheme was used in our GAMD simulations. The sigma0P and sigma0D parameters for the GAMD simulation were set to their default values (6.0). All GAMD simulations included a 60 ns equilibration phase before the "production" part. In the first 10 ns, no GAMD potential was applied on the system and the 4-10 ns part was used for data

collection. In the next 50 ns the GAMD potential was applied on the system. The GAMD parameters were updated at regular intervals except for the first 5 ns of the 50 ns phase.

The production GAMD simulations were performed on 310 K under NVT conditions. The Langevin thermostat was applied in the simulation, with a gamma constant of 2.0. In the production simulations the Monte Carlo barostat was used for pressure coupling. Long-range electrostatic interactions were computed using the PME method. A Coulomb cutoff of 12 Å was used in the simulations. A force switch was applied between 10 Å and 12 Å for the Lennard-Jones interactions, recommended for CHARMM force fields. All production simulations were 600 ns long. For each mutant, two parallel simulations were run for the 1T1F-based and three for the 1NQ9-based systems.

The CPPTRAJ software was used for most trajectory analysis, including RMSD and RMSF calculations as well as clustering. “Generalized correlation” calculations were performed using a method developed by Lange and Grubmüller, based on an earlier work by Ichiye and Karplus.

3.13.2. In Silico Prediction of the Consequences of Missense Mutations

To predict whether the seven missense mutations in the present study are dis-ease-causing, we used five different tools available as web servers, resulting in 6 different scores in total. PolyPhen2 is a tool for the prediction of the consequences of amino acid substitutions in human proteins. It takes into account information from multiple sources: homologous sequences, structural features and 3D structures. PolyPhen2 uses two different models trained on different datasets, HumDiv and HumVar. HumVar is better suited for detecting mutations with drastic, disease-causing effect, while HumDiv is in-tended for predicting more complex phenotypic effects and mildly deleterious variants. MutPred2 uses a machine learning model for classifying mutations in humans as pathogenic or benign. It can also predict the impact of the mutation on several structural and functional properties of the protein. PhD-SNP is a Support Vector Machine (SVM)-based classifier for missense mutations that takes into account the mutation, the sequence environment and the sequence profile.

SIFT is a sequence-homology-based method for distinguishing between amino acid substitutions in proteins that with or without a phenotypic consequence. Mutations with a score below or equal to 0.05 are classified as damaging. MutationTaster is a tool that predicts the consequences of variants and mutations in DNA sequences. In MutationTaster, the score is the probability of correct prediction and not a value that distinguishes between pathogenic and non-pathogenic mutants. For the PolyPhen2, Mut-Pred2 and PhD-SNP methods,

mutations with a score above a cut-off of 0.5 were considered pathogenic. For the two missense mutants located in the signal peptide, p.Arg14Lys and p.Cys32Tyr, we also performed signal peptide cleavage prediction using SignalP 6.0, for eukaryotic signal peptide only.

4. RESULTS

4.1. Results of examination on ATBp3, AT Basel and AT Padua mutations

4.1.1. Clinical and Laboratory Characteristics of Patients with Antithrombin Type II Heparin-binding Site Mutations

Until recently, we have diagnosed n=19 individuals with AT Basel, n=31 individuals with AT Padua. Most of our patients, however, carried the ATBp3 mutation and both homozygotes (n=52) and heterozygotes (n=239) have been found. Venous thrombosis was registered in almost all ATBp3 homozygotes (88.8%) and it was also relatively frequent in ATBp3 heterozygotes, while a great proportion of AT Basel and AT Padua patients were free from venous thrombotic symptoms at the time of data collection. A few patients in the ATBp3 and AT Padua groups suffered from pulmonary embolism without having obvious embolic source, and they were not diagnosed with patent foramen ovale, either. Arterial thrombosis was the most frequent in AT Basel. The frequency of pregnancy complications, as pre-term birth or spontaneous abortions was slightly higher in AT Padua patients, however the difference was not statistically significant. Median of hc-anti-FXa AT activity was obviously the lowest in ATBp3 homozygotes and the Innovance AT method, which was used for the measurements, gave AT activity results below the cut-off value in all AT Basel and Padua mutations also. There were a few patients (n=4) with hc-anti-FXa AT activity slightly above the cut off value of 80%. The p-anti-FXa AT activity in the absence of heparin in the reagent was significantly lower in both heterozygous and homozygous ATBp3 mutants as compared to AT Basel and AT Padua. Ratio of p-anti-FXa AT to hc-anti-FXa AT activity was the highest in ATBp3 homozygotes demonstrating the largest effect of this genotype on AT-heparin interaction. AT antigen concentration was significantly lower in the ATBp3 groups when comparing it to the other two mutants. The crossed immunoelectrophoresis (CIE) results in the plasma of patients with different mutations demonstrated a heparin low affinity fraction and a heparin high affinity fraction in all of the heterozygote plasmas. In the case of ATBp3 homozygote plasma only heparin low affinity AT could be visualized.

The less anodal abnormal peak in the AT Padua heterozygote plasma was larger than the normal peak. This phenomenon is concordant with the previous results of Girolami et al..

4.1.2. Thermostability of Wild Type and Budapest 3 Homozygous Antithrombin

In nano DSF (Differential Scanning Fluorimetry) experiments comparing the ATBp3 homozygous mutant and wild type (WT) AT separated from human plasma (n=3 independent measurements) we observed significant differences. Change in ratio of tryptophan fluorescence at 350 nm to 330 nm with respect to increase in temperature was used to characterize the thermal stability of WT and ATBp3 mutant AT proteins. Two parameters, the onset temperature (Tonset) of denaturation and the transition midpoint (Tm) were determined using the melting curves of the proteins. In the case of the WT AT the Tonset and Tm values were 46.2±1.3°C and 57.6±0.1°C, respectively, while for the mutant AT the Tonset was 42.7±1.47°C and the Tm was 57.1±0.03°C. Both Tm ($p=0.0031$) and Tonset ($p=0.0371$) values were significantly lower in case of the ATBp3 mutant compared to WT AT indicating a lower thermostability for the mutant protein.

4.1.3. Investigation of Heparin-binding Characteristics of Wild Type and Different IIHBS Antithrombin Mutants

As for AT Basel and AT Padua only heterozygote patients exist (homozygosity is suggested to be lethal) we investigated the heparin-binding characteristics of different type IIHBS mutants in a purified system using recombinant proteins. Upon surface plasmon resonance (SPR) experiments data obtained at six different AT concentrations were averaged for each mutant. As it was expected, the strongest AT-heparin binding was observed in the presence of the WT AT protein (KD = 6.4 x 10-10 M, KA = 2.2 x 109 1/M). The association rate constant (ka) was the highest (ka = 1.37 x 107 1/Ms) for WT AT among the investigated purified recombinant AT proteins. These data suggest that the formation of the AT-heparin complex occurs most rapidly in case of WT AT. The dissociation rate constants in all mutants were of the same order of magnitude (10-3). (For the WT AT kd = 6.75 x 10-3 1/s.)

Out of the mutant AT proteins, ATBp3 formed the most stable complex with heparin (KD = 2.15 x 10-8 M, KA = 1.62 x 108 1/M) as well as complex formation was the fastest (ka = 3.25 x 105 1/Ms). The dissociation rate constant for ATBp3 mutant was kd = 2.47 x 10-3 1/s. As compared to the data obtained for the WT protein, ATBp3 appeared to exhibit a significantly weaker interaction with heparin, with a slower association rate. SPR studies performed on WT and ATBp3 homozygous mutant proteins isolated from human plasma also showed approximately two orders of magnitude differences in KD values (data not shown).

For the AT Basel mutant, the association/dissociation kinetic and equilibrium parameters were as follows: ka = 1.03 x 104 1/Ms and kd = 4.45 x 10-3 1/s; KD = 7.64 x 10-7 M and KA

= 2.40 x 106 1/M. These data show that AT Basel binds to heparin more slowly than WT AT and ATBp3 and the association constant of AT Basel is almost 1 order of magnitude lower than that of ATBp3.

Among all mutants, AT Padua had the weakest interaction with heparin (KD = 1.08 x 10-6 M, KA = 2.37 x 106 1/M) and it had the slowest complex formation (ka = 1.01 x 104 1/Ms). However, the dissociation rate constant (4.51 x 10-3 1/s) was almost equal to the kd values obtained for the other mutants.

4.1.4. In silico Modeling of Wild Type and Different Type IIHBS Mutant Antithrombin Proteins

Conformation of the 22-46 Loop

The 22-46 loop, close to the heparin binding site, has been suggested to play a role in the control of AT pentasaccharide binding. This loop is highly flexible, and many of its amino acids are not resolved in X-ray diffraction structures. Despite the lack of experimental data, enhanced sampling MD simulations allowed us to study the conformation of this loop. As molecular modeling was performed using the structure of the mature protein the numbering in the sections of in silico experiments follows the classical way, ie. the first aminoacid in the mature protein is numbered as +1. (ATBp3 corresponds to Leu99Phe, AT Basel corresponds to Pro41Leu and AT Padua corresponds to Arg47His.)

Among the amino acids affected by the mutations studied, Pro41 (position of AT Ba-sel) is located in the 22-46 loop, thus, the conformation of this loop may be an important factor in the reduced heparin affinity of this variant.

In the two simulations of the non-activated AT Basel variant (Pro41Leu), two major types of conformations were observed. In the first type, the loop occupied a position very close to the heparin-binding site, forming salt bridges and hydrogen bonds with the aminoacids of the site. In the second type, mostly observed in the other 600 ns trajectory, the distance from the heparin-binding region was large. This suggests a significantly increased conformational variability of the loop compared to the WT, with some conformations potentially interfering with the binding of a heparin pentasaccharide.

However, in the pentasaccharide-bound AT simulations, no close contact was observed between the loop and the heparin-binding region. This suggests that the altered conformation of this loop probably has no or only small destabilizing effects on the pentasaccharide binding once a “strong” complex has been formed. The highly charged nature of the pentasaccharide

and the altered electrostatic effects could prevent the loop from transitioning into the alternative conformation.

DSSP (Define Secondary Structure of Proteins) Analysis of the N-terminal Part of the Antithrombin Protein

We analyzed the changes of secondary structure in the N-terminal-part (amino acids 1-145, this includes the entire heparin binding site, but not the "A" beta sheet) using the DSSP method, as implemented in the CPPTRAJ software. This analysis was performed for both the "not activated AT" (1T1F) and "AT-pentasaccharide complex" (1NQ9) systems.

Regarding the 1T1F-based systems, conformational changes were evident in the AT Basel simulations in the region described in the previous section as compared to the WT AT. However, no significant conformational changes were visible in the heparin-binding regions, including helix P (amino acids 112-120) (For the discussion of helix P conformation in GAMD simulations, see reference). Among the "complex" simulations, the AT Padua (Arg47His) variant (and especially its neutral from) showed conformational changes in the 30-35 region. Similarly to the 1T1F-based simulations, there were no significant conformation changes in the heparin-binding region. Some elongation of helix D was visible in the ATBp3 (Leu99Phe) trajectories as compared to the wild type.

Root Mean Square Fluctuations Analysis

Root mean square fluctuations (RMSF) are common analysis methods to describe the flexibility of amino acids in the protein. It can be used for measuring the increases in fluctuation caused by a missense mutation. Additionally, conformational changes can cause increases or decreases in the fluctuations at specific parts of the molecule.

Among the "not activated" AT simulations, the ATBp3 variant showed moderate increases in fluctuations as compared to the wild type protein and the AT Basel variant. We could observe increased fluctuations both in parts of the protein close to the affected amino acid (residues 50-100), as well as in more distant regions (amino acids 320-330, 360-380, 420-430). However, the increases in fluctuations are minor in other parts of the tertiary structure. These findings are consistent with a variant in which the stability of the native conformation was moderately decreased, but still secreted in considerable amounts. In case of the AT Basel variant, increases in fluctuation was mainly observed in the 110-130 and 300-320 regions. The most interesting finding, however, was the highly increased fluctuations in the AT Padua variant. This increase was observed in both protonation states of residue 47. The increases are

particularly large in the region close to the F helix (180-210) and in the helices in the 290-310 region. This suggests that this variant may affect the conformation and also the allostery in distant parts of the molecule.

Concerning the heparin-activated state, the RMSF values are expected to reflect the change in the stability of the tertiary structure, similarly to the non-activated state. However, conformation changes in the regions with higher-than-average fluctuations, known to be involved in the allosteric mechanism, can also trigger an increase in the RMSF. Regions that participate in this process include the C-terminal end of the D helix, the reactive center loop and the exosite interacting with FXa and FIXa. The ATBp3 variant showed increases in fluctuations compared to the WT, similarly to the "non-bound" state. The C-terminal end of helix D (amino acids 125-135) plays an important role in the allosteric activation. In case of the WT system, significant decrease was observed for the "bound" state as compared to the 1T1F-based simulations of non-activated WT AT. However, this was not the case for ATBp3 and AT Basel variants; the RMSF values were still high in both states. In case of the AT Padua variant the increased fluctuations, which were observed in the 1T1F-based systems representing non-bound AT, could be observed also in the heparin-bound simulations of this variant. The regions with the largest increases are nearly the same in the two types of systems for AT Padua mutation.

Analysis of Allosteric Pathways

The conformational activation of AT is essentially an allosteric process in which the heparin-binding site, the reactive center loop, and the coagulation factor binding exosites are involved. Like in our previous work, the allosteric pathways were investigated using a method developed by Lange and Grubmüller, which can detect correlated motions in MD trajectories. We performed this analysis for both the 1T1F and the 1NQ9-based systems.

In general, the AT Basel variant showed similar patterns to the WT AT, indicating that this mutation probably has only relatively small effects on allostery. As a contrast the patterns were different in case of the ATBp3 and especially the AT Padua mutations. This likely corresponds to significant alterations in the allosteric processes.

Root Mean Square Deviations of Pentasaccharide Binding

The Root Mean Square Deviations (RMSDs) of the pentasaccharide ring and inter-glycosidic atoms compared to the X-ray diffraction structure were used to describe the conformation of the ligand. There was no significant difference between the pentasaccharide RMSD values

observed in the WT, AT Basel and ATBp3 simulations. In contrast, the AT Padua protonated variant showed increased pentasaccharide RMSD values as compared to all other simulations. Surprisingly, this decrease in the binding strength could only be observed in the protonated variant.

Full or nearly full dissociation of the ligand was not observed in any of the simulations for a certain time period. It should be noted, however that all variants investigated had a glycosylation pattern corresponding to β-AT that likely resulted in a stronger AT-heparin interaction. The reason for using β-AT in the simulations was the difficulty of the proper conformational sampling of a large oligosaccharide in α-AT close to the binding site. Very extensive MD simulation would be required to get a proper "average" for the interaction between the glycan and the pentasaccharide.

4.2. Results of examination on the nine new antithrombin mutations

4.2.1. Clinical and Laboratory Characteristics of Antithrombin-Deficient Patients

Patients with ATD were diagnosed at our tertiary center for hemostasis diseases between 2016 and 2021. Altogether, n = 311 patients were genetically investigated for ATD during this period. The majority of them carried the founder AT Budapest3 (p.Leu131Phe) mutation. In our cohort, however, we have found nine mutations within SERPINC1 that have not been reported before. These mutations are missing from the different databases (HGMD Professional and ClinVar, https://www.ncbi.nlm.nih.gov/clinvar, last accessed 11th November, 2023), or they have been reported by us. We filtered our results against data deposited in the 1000 Genomes Project (http://www.1000genomes.org, last accessed 11 November 2023), and they were not considered polymorphisms. All patients were heterozygous for these novel mutations. Two patients (father and son) carried the c.41G>A (p.Arg14Lys) mutation. In their plasma samples, decreased heparin co-factor anti-FXa AT activity (hc-antiFXa) and decreased progressive anti-FXa AT activity (p-antiFXa) were measured. The AT antigen concentration was also decreased, suggesting a rather quantitative ATD. The proband suffered a DVT at the age of 16 years, and no recurrence was registered. His father, a carrier of the same variant, has not reported a thrombotic episode as yet. The index patient was recommended to receive long-term VKA treatment; his father is not anticoagulated.

The second mutation (c.95G > A, p.Cys32Tyr) was carried by one female patient, who suffered her first thrombotic episode, a DVT with PE, at the age of 20 years during pregnancy. We registered two recurrences: a mesenterial vein thrombosis occurring at the age of

41 years and a PE several years later. The patient was on VKA after the first and second thrombosis for one year each; however, after the third thrombotic episode, she was put on lifelong anticoagulation, starting with VKA and then switching to rivaroxaban (20 mg OD). The patient's family history is positive for thrombosis: her three brothers also suffered from DVT, and two of them had a fatal PE. However, they were not investigated for thrombophilia at that time. The patient's daughter suffered from DVT after delivery. The hc-antiFXa and p-antiFXa AT activity values of the patient were low, and a proportionally decreased AT antigen level was measured.

The third mutation (c.232C > G, p.Arg78Gly) affects the heparin-binding region of AT. The carrier was a young female patient with endometriosis. She was examined due to infertility, but she had never had a thrombotic episode. Her family history was also negative for thrombosis. Before an in vitro fertilization (IVF) procedure, she was diagnosed with ATD. During the IVF procedure and her pregnancy, she was treated by LMWH, but no long-term anticoagulation was indicated. The hc-antiFXa AT activity of the patient was decreased, while normal p-antiFXa AT activity and a normal AT antigen level were detected suggesting type II HBS AT deficiency.

The fourth mutation (c.362T > G, p.Met121Arg) was carried by a young female who had had one episode of DVT at the age of 34 years. Hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low, suggesting type I ATD. The patient was put on long-term VKA anticoagulation.

The fifth mutation (c.734T > C, p.Leu245Pro) was carried by a male patient with proximal DVT at the age of 42 years. He had no provoking factors in his case history. Hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low, suggesting type I ATD. Upon diagnosis, he was put on warfarin for long-term anticoagulation. No recurrent thrombotic events were registered.

The sixth mutation (c.809delT, p.Leu270Argfs*14) was detected in a male patient who had had 5 episodes of DVT. His first DVT was diagnosed at the age of 16 years. From the clinical point of view, this case showed the most severe thrombotic phenotype. Hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low, suggesting type I ATD. The patient is on lifelong anticoagulation and is currently taking rivaroxaban (20 mg OD).

The seventh mutation (c.1349A > T, p.Asn450Ile) was carried by a family with four members, of whom two had already suffered from DVT at the ages of 23 and 40 years. Their hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low,

suggesting type I ATD. Patient 7_1 has not suffered from thrombosis, as yet; however, she has had four pregnancies, among which the first ended with a spontaneous abortion. During the three subsequent pregnancies, the patient was treated with LMWH and AT concentrate, and those pregnancies were successful. No long-term anticoagulation was introduced for her. The sister of the index patient (7_2) was also treated with LMWH and AT concentrate during pregnancy; however, due to DVT in her case history, she was put on long-term VKA anticoagulation. Patient 7_3 is also anticoagulated with VKA, while patient 7_4 is not, and he has not suffered from thrombosis, as yet.

The eighth mutation (c.1367-1368delGCinsCTACA, p.Gly456delins_Ala-Thr) was detected in a female patient who had had a pregnancy-associated DVT at the age of 27 years, which was followed by a second (unprovoked) episode 5 years later. Hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low, in accordance with a type I ATD. The patient is on lifelong anticoagulation with apixaban (5 mg BID).

Finally, the ninth mutation (c.1381C > A, p.Pro461Thr) was detected in three unrelated patients. One of them had suffered an unprovoked thrombosis followed by pulmonary embolism at a relatively old age (62 years), and he is now on VKA treatment; the other two patients were much younger at the time of their thrombotic events, but both DVTs were provoked. Both patients are on DOACs (apixaban 5 mg BID and rivaroxaban 20 mg OD). Hc-antiFXa and p-antiFXa AT activity values were disproportionally low compared to AT antigen values, suggesting a rather functional AT deficiency.

Concerning laboratory investigations, the basic tests of coagulation were normal for all patients, or at least appropriate for their anticoagulant treatment. Thrombophilia testing showed activated protein C resistance, FV Leiden heterozygosity in two patients and no other detected thrombophilia.

4.2.2. AT Sequence Homology Study with UniProt Database

As indirect evidence of pathogenicity, it is known that disease-causing mutations are found in the strongly conserved positions of proteins.

Therefore, we examined AT sequence homology in seven different species at the positions of the nine mutations using the UniProt Database. The investigated amino acid positions are identical in the seven different species (except at position 270, where there is a leucine instead of a proline in three different species; however, they are similar non-polar amino acids with hydrophobic side chains), meaning strongly conserved positions. In the case of P. troglodytes, we noted that all positions and their surrounding regions are identical. Position 78 and 121

and their surrounding regions are identical in all seven species. The signal peptide sequence is heterogeneous in the seven species, but it has three conserved parts: a hydrophilic part in the N terminal, a hydrophobic core and a polar C-terminal region. Position 32 is in the conserved polar C-terminal region containing the signal peptide cleavage site.

4.2.3. In Silico Prediction of the Consequences of Missense Mutations

We analyzed the seven AT missense mutations using six different tools available as web servers that can predict their consequences. The mutations p.Met121Arg, p.Leu245Pro, p.Asn450Ile and p.Pro461Thr were predicted by all six methods to be pathogenic or disease-causing. The results for the p.Arg78Gly mutant, affecting an amino acid close to the heparin binding site, were inconclusive. It was classified as benign by PolyPhen 2 (both scores) and PhD-SNP but as pathogenic by all other tools. Two missense mutations, p.Arg14Lys and p.Cys32Tyr, are located in the signal peptide sequence of AT. PolyPhen2 and SIFT predicted these mutants to be deleterious, while Mut-Pred2 and MutationTaster designated them as non-pathogenic. PhD-SNP classified the first mutant as benign and the second as disease-causing. We performed an in silico analysis of signal peptide cleavage using SignalP 6.0. for the two mutations located in this sequence. SignalP 6.0 predicted a signal peptide cleavage site between amino acids 32 and 33 for the wild-type protein and the same situation for the p.Arg14Lys mutant, and the prediction indicated high confidence (>0.999). For p.Cys32Tyr, the program still detected a cleavage site after position 32, but the confidence of the prediction was rather low; it was only slightly above 0.5 (0.542).

4.2.4. Detection of Wild-Type and Mutant Antithrombins in the Cell Lysates and in the Media of Trans-fected Cells

WT AT appeared as a clear band in the cell lysates and in the conditioned media of HEK293 cells at 58 kDa. The positive control band represented AT from the pooled plasma of 5 healthy individuals and appeared as a single band. As expected, no signal from mock transfection could be detected. In the media of transiently transfected HEK293 cells, we detected a clear band at 58 kDa in the cases of p.Arg14Lys, p.Arg78Gly and p.Pro461Thr. However, only a faint band, if any, could be visualizes for p.Cys32Tyr, p.Met121Arg, p.Leu245Pro, p.Leu270Argfs*14, p.Asn450Ile and p.Gly456delins_Ala_Thr. In the cell lysates of transiently transfected cells, AT could be visualized in the cases of p.Arg14Lys, p.Arg78Gly, p.Met121Arg, p.Leu245Pro, p.Gly456delins_Ala_Thr and p.Pro461Thr. In the

cases of p.Cys32Tyr, p.Leu270Argfs*14 and p.Asn450Ile, AT was practically absent or showed a very faint band (Figure 2A).

Based on the Western blot analysis, we classified the mutants into three groups. The first group included the cases of p.Cys32Tyr, p.Leu270Argfs*14 and p.Asn450Ile, where we detected low AT protein expression both in the conditioned media and in the cell lysates, suggesting a low level of protein synthesis or even a lack of mRNA. In the case of p.Asn450Ile, Western blotting indicated a reduced (approximately 56 kDa) molecular weight for the AT protein. The second group (p.Met121Arg, p.Leu245Pro and p.Gly456delins_Ala_Thr) showed high AT protein expression in the cell lysates but low AT protein expression in the media, suggesting a secretion disorder. The third group (p.Arg14Lys, p.Arg78Gly, p.Pro461Thr) included those AT proteins appearing as strong bands both in the cell lysates and in the media. These mutants are suggested to be functional variants, or they may have a minor effect on AT structure and function .

In the cases of six mutations (p.Arg14Lys, p.Cys32Tyr, p.Arg78Gly, p.Met121Arg, p.Leu245Pro and p.Pro461Thr), stable transfection was also performed in order to gain higher protein concentrations for further investigations (see later). To check the quality of stable transfection, we detected the WT and the six mutant AT proteins by Western blotting in the conditioned media. The appearance of the bands corresponding to the different mutants was identical to that seen in the Western blot of the transient transfection.

4.2.5. Real-Time Quantitative PCR Analysis

RT-qPCR was performed to determine the expression of AT mRNA in the transiently transfected HEK293 cells in three independent transfections. To detect the efficiency of transfection, we measured β-GAL mRNA expression. The relative quantification of target RNA was achieved by the comparative threshold cycle (CT) method, and the target CT numbers were normalized to OAZ, a housekeeping gene. The mRNA contents of the variants were expressed as values of the ratio relative to WT, and they were as follows: p.Arg14Lys 1. 09 ± 0.03; p.Cys32Tyr 1.03 ± 0.04; p.Arg78Gly 1.03 ± 0.04; p.Met121Arg 1.00 ± 0.03; p.Leu245Pro 1.01 ± 0.01; p.Leu270Argfs*14 0.99 ± 0.07; p.Asn450Ile 0.94 ± 0.04; p.Gly456delins_Ala_Thr 0.96 ± 0.05 and p.Pro461Thr 0.98 ± 0.03, respectively. (Data represent the relative quantification (RQ) ± SEM of each transcript normalized to OAZ.) The AT mRNA expression levels of the nine mutants were not significantly different from that of AT-WT according to RT-qPCR. These results suggest that in the case of decreased AT, even in the cell lysates, the mutations do not affect the mRNA level. In the case of p.Cys32Tyr,

p.Leu270Argfs*14 and p.Asn450Ile mutations, for which little or no AT was detected in the cell lysates, a defect in protein synthesis is therefore more likely.

4.2.6. AT Antigen and Activity of Mutant AT Proteins Expressed in HEK293 Cells

WT and mutant AT antigen levels were determined by ELISA in duplicates from each of the different transfection reactions. AT antigen values were normalized to the transfection efficiency by performing β-GAL measurements.

The AT antigen concentrations in the conditioned media were as follows (number of different transfection reactions in brackets): p.Arg14Lys 131.00 ± 16.39% (n = 4); p.Cys32Tyr 26.25 ± 5.72% (n = 4); p.Arg78Gly 141.75 ± 29.77% (n = 4); p.Met121Arg 210.00 ± 92.11% (n = 4); p.Leu245Pro 100.75 ± 45.55% (n = 4); p.Leu270Argfs*14 28.33 ± 9.56% (n = 3); p.Asn450Ile 27.00 ± 8.50% (n = 3); p.Gly456delins_Ala_Thr 43.66 ± 17.42% (n = 3) and p.Pro461Thr 147.00 ± 30.09% (n = 4), respectively.

The AT antigen concentrations in the cell lysates were as follows (number of different transfection reactions in brackets), p.Arg14Lys 188.25 ± 11.69% (n = 4); p.Cys32Tyr 31.33 ± 0.88% (n = 3); p.Arg78Gly 134.25 ± 13.14 % (n = 4); p.Met121Arg 1706.25 ± 585.45% (n = 4); p.Leu245Pro 1555.75 ± 568.72% (n = 4); p.Leu270Argfs*14 0.00 ± 0.00% (n = 3); p.Asn450Ile 20.00 ± 6.55% (n = 3); p.Gly456delins_Ala_Thr 64.66 ± 18.67% (n = 3) and p.Pro461Thr 262.50 ± 67.22% (n = 4), respectively.

As can be seen, AT antigen concentration was low in the cases of p.Cys32Tyr, p.Leu270Argfs*14 and p.Asn450Ile mutants both in the cell lysates and in the media, corresponding to the results obtained by Western blotting. A considerable amount of AT was detected in the cell lysates of the other six mutants. Among them, the AT antigen was very high in the cases of p.Met121Arg and p.Leu245Pro. AT antigen concentrations in the media of the p.Arg14Lys, p.Arg78Gly and p.Pro461Thr groups were similar to that of WT AT; in the case of p.Gly456delins_Ala_Thr, it was low; and in case of p.Met121Arg and p.Leu245Pro, AT antigen concentrations were disproportionally low in the media compared to the AT level in the corresponding cell lysates.

Except for those mutants where the AT antigen concentration was low in the media, heparin cofactor activity and progressive activity from the conditioned media of the transfected cells were determined by amidolytic assay from at least two independent transfections (Figure 3B). The heparin cofactor activity and progressive activity results of the mutants are represented as percentages of WT AT activity, which was considered 100%. The hc-antiFXa values were as follows: p.Arg14Lys 85.16 ± 18.99%; p.Arg78Gly 61.50 ± 8.50%; p.Met121Arg 106.5 ±

26.67%; p.Leu245Pro 110.00 ± 25.07%; and p.Pro461Thr 93.75 ± 44.67%. The p-antiFXa values were as follows: p.Arg14Lys 99.56 ± 30.07%; p.Arg78Gly 67.54 ± 59.85%; p.Met121Arg 133.46 ± 46.10%; p.Leu245Pro 120.14 ± 43.47% and p.Pro461Thr 160.27 ± 67.06%. In the cases of the p.Arg14Lys, p.Met121Arg and p.Leu245Pro mutants AT activity did not show a decrease in the heparin cofactor and progressive assays, and the two assays gave proportional results. In the case of the p.Arg78Gly mutant, AT heparin cofactor activity was only 60% of the wild-type value, and progressive AT activity values showed a large variance, having reduced and normal values as well in the different experiments. In case of p.Pro461Thr, a large difference between hc-antiFXa and p-antiFXa values was demonstrated.

4.2.7. Investigation of p.Arg78Gly and p.Pro461Thr Mutant AT Heparin Binding by Surface Plasmon Resonance

Based on the functional assays, altered heparin binding was hypothesized in the cases of p.Arg78Gly and p.Pro461Thr AT. Therefore, we investigated the heparin-binding characteristics of these two mutants and used WT AT as a control in a purified system using recombinant proteins. The kinetic and affinity parameters of the interaction were calculated based on the sensorgrams obtained at different AT concentrations and averaged for each AT. As expected, the strongest AT–heparin binding was observed in the case of WT protein (KD = 8.49×10^{-7} M). The association rate constant (ka) was ka = 2.66×10^{6} 1/Ms. These data suggest that the formation of the AT–heparin complex occurs most rapidly in the case of WT AT. In the case of the p.Arg78Gly protein, the KD value was 1.69×10^{-6} M, and the association rate constant (ka) was ka = 1.15×10^{3} 1/Ms. In the case of the p.Pro461Thr protein, KD was 1.61×10^{-6} M, and the association rate constant (ka) was ka = 1.38×10^{4} 1/Ms. Based on these results, AT–heparin complex formation seems to be much slower in the case of the mutant proteins. Both mutants showed weaker interaction with heparin, and their Ka and Kd values were similar. The dissociation rate constants (kd) in the WT and all mutants were on the same order of magnitude (10^{-3}). These observations support the presence of an altered heparin interaction for both mutants.

4.2.8. N-Glycosidase F Digestion of Mutant AT Proteins

As N-linked glycosylation is a crucial post-translational modification involved in protein folding, we used N-glycosylation as a tool to study mutant AT proteins. N-Glycosidase F cleaves asparagine-bound N-glycans from glycoproteins. AT has four N-glycosylation sites (Asn128, Asn167, Asn187 and Asn224); missense variants of these sites and others were

reported to inhibit glycosylation. N-Glycosidase F digestion was carried out in the cases of AT variants with low amounts of AT in the cell media, suggesting a secretion defect; these variants comprised p.Met121Arg, p.Leu245Pro, p.Asn450Ile and p.Gly456delins_Ala_Thr. Treatment of conditioned media with PNGase altered the electrophoretic migration patterns of the WT and all four variant AT proteins, indicating that these mutant proteins were N-glycosylated in the ER. The WT and the four variant AT proteins had identical electrophoretic migration patterns. Non-digested AT migrated as a single band on SDS-PAGE, with a molecular weight of 58 kDa; PNGase-digested AT was also detected, with a molecular weight of 50 kDa.

5. DISCUSSION

5.1. Discussion of results of ATBp3, AT Basel and AT Padua mutations

The recognition of type IIHBS AT deficiency is dated back to the eighties, when several observations on abnormal AT-heparin interaction were published. AT Basel was associated with arterial thrombosis in the early report of Brennan, and the mutant AT showed an abnormal peak in heparin-Sepharose affinity chromatography. In a monoclonal antibody-based heparin-binding affinity assay AT Basel demonstrated 40-fold reduction in heparin-binding affinity comparing it to normal AT. AT Padua showed 30-fold decrease in heparin affinity in another study using other method for investigation. The ATBp3 variant showed reduced heparin affinity by heparin-Sepharose chromatography, and the antiproteinase activity was decreased as compared to normal AT in the presence of unfractionated heparin (UFH) or the AT-binding pentasaccharide. According to the results of an elegant study of Martinez-Martinez et al. plasma samples of ATBp3 homozygous patients showed an increased fraction of AT with low heparin affinity. The major finding of that study was the demonstration of the compensatory role of β-AT in the type IIHBS mutants ATBp3 and AT Basel. They did not investigate AT Padua, however in case of an Arg to Cys mutation in the same position the recombinant β-isoform could compensate for the strong effects of this mutation in the interaction with heparin. Direct comparison of the heparin-binding features of ATBp3, AT Basel and AT Padua by using uniform methodology for all mutants, however, has not performed, as yet. As AT Basel and AT Padua homozygous patients do not exist, only recombinant, purified systems yielding high amount of homozygous mutant AT variants are able to serve as basis of biochemical studies. Complex in silico studies comparing the behavior of these type IIHBS mutants have not been executed, either.

In our relatively large AT deficient patient group (n=449 patients), the type IIHBS subtype is the most common. We demonstrated that the frequent occurrence of the ATBp3 mutation is the result of a founder effect in this AT deficient population. Several clinical studies suggested differences in the clinical and laboratory phenotypes, especially in the strength of AT-heparin binding among the different mutants resulting in type IIHBS AT deficiency. The type IIHBS ATBp3, AT Padua and AT Basel mutations were identified in association with VTE, ATE and pregnancy complications as well.

The laboratory characteristics of our IIHBS AT deficient patients were heterogeneous. The hc-anti-FXa AT activity was decreased. The p-anti-FXa AT activity was low normal or it was in the normal range. The AT antigen levels were normal in AT Basel and in AT Padua patients. In the case of ATBp3 homozygous and heterozygous individuals, however, the AT antigen concentration was significantly lower upon comparison it to that of AT Basel and AT Padua. This suggests mild quantitative defect (besides heparin-binding abnormality) in ATBp3 mutation, which may be caused by protein instability. According to the results of our nanoDSF experiments, homozygous ATBp3 purified from plasma exerted decreased thermostability as compared to WT AT. (As no homozygous AT Basel and AT Padua plasmas were available this experiment was not done in these mutations.) ATBp3 instability was also demonstrated by our in silico studies, where elongation of helix D was observed in its native form and increased RMSF was found in its non-activated state. These findings, although don't seem to be deleterious, but may lead to mild secretion defect or may cause increased elimination of ATBp3 AT due to its instability. According to our knowledge no pulse-chase experiments have been performed to confirm these hypotheses to date, however in two studies mutations in AT gene close to the ATBp3 position led to decreased secretion.

In this study we focused on the AT-heparin binding characteristics of ATBp3, AT Basel and AT Padua mutants and intended to explore the background of differences in their heparin-binding affinity.

Pooled normal human plasma and AT deficient plasma from ATBp3 heterozygous, ATBp3 homozygous, AT Basel heterozygous and AT Padua heterozygous patients were examined by CIE. As it was visualized on the electrophoresis the heparin binding affinity in the case of ATBp3 heterozygous and AT Basel heterozygous plasmas did not differ as much. By examining AT Padua heterozygous plasma a stronger low heparin affinity fraction was observed, while in ATBp3 homozygous plasma only this low heparin affinity fraction

appeared. These observations suggested AT Padua as the most severely affected AT mutant from the point of view of heparin affinity.

Due to the lack of AT Basel and AT Padua homozygous subjects a direct comparison of the biochemical consequences of these mutations in plasma samples is problematic. We therefore established recombinant models for further investigations. The recombinant WT AT and ATBp3, AT Basel, AT Padua proteins were expressed in HEK293 cells. The stable expressed proteins were harvested, concentrated and purified by affinity chromatography. These purified proteins were examined by SPR. We investigated the binding affinities of WT and mutant AT to immobilised heparin surface. SPR has several advantages among methods used for testing molecular interactions. It allows label-free, real-time, medium-throughput tests and requires only a small amount of materials and reagents. It uses an optical method to measure the change in refractive index of the medium close to the gold surface to monitor the binding of analyte molecules to ligand molecules, which are immobilized on the metal surface. Only a very few studies have been published in which AT-heparin interaction was investigated by SPR technique, as yet. Moreover no study exists with a direct comparison of type IIHBS mutants. A novel method has been developed for the easy measurement of heparin's anticoagulant activity using SPR by Zhao et al.. The anticoagulant activity of target heparin was evaluated by measuring the competitive AT binding of analyte heparin in the solution phase and USP heparin immobilized on chip surface. Heparins, obtained from different animal sources, and low molecular weight heparins were analyzed. The results were reproducible and correlated well with the results of chromogenic assays (correlation coefficient r = 0.98 for anti-Xa and r = 0.94 for anti-IIa) used in routine hemostasis laboratories. This well designed competition SPR method has been previously used in many studies for characterizing heparin-protein interaction. Biomolecular interaction analysis using SPR was utilized to record and analyze prothrombin, thrombin, AT, and fibrinogen heparin binding properties in real time. Biotinylated heparin, heparin–albumin conjugate, and albumin were immobilized onto streptavidin-coated sensors as ligands, respectively. The binding pattern of AT to both heparin and heparin–albumin conjugate, although specific, was biphasic, possibly due to a conformational change during the binding process. Steady-state kinetic analysis revealed a KD value of $281\pm24\times10^{-9}$ M for the heparin surface. For the conjugate surface, a KD of $53\pm5\times10^{-9}$ M was calculated, indicating a higher affinity toward heparin–albumin conjugate. As compared to our results, these KD values are closest to the KD values obtained for our recombinant WT AT (KD = 6.4 x 10^{-10} M). In our experiments AT Padua had the lowest heparin affinity (KD = 1.08 x 10^{-6} M) and KD value for ATBp3

mutant AT (KD = 2.15 x 10^{-8} M) was the closest to that of WT AT, however there was still a two-magnitude difference. Heparin affinity of AT Basel was in between (KD = 7.64 x 10^{-7} M).

We intended to explain the differences observed in the biochemical studies by means of in silico analysis of WT AT and mutant AT proteins. In the simulations of AT Basel variant without a ligand, we could observe a novel conformation of the 22-46 loop, which can probably interfere with the binding of the pentasaccharide. Among the variants studied, AT Basel mutation affects the fluctuations and allosteric pathways the least as compared to the WT AT, therefore it is unlikely that this mutation has strong destabilizing effect on the secondary and tertiary structures. For the ATBp3 variant, we could observe increased fluctuations in the protein, both close to the heparin-binding site as well as in more distant regions. From the "generalized correlation" calculations, we can conclude that this variant likely affects the allosteric pathways that are involved in the conformational activation of AT. According to our simulations, the AT Padua variant has the most severe consequences. The fluctuations as measured by the RMSF of α-carbon atoms were significantly increased comparing it to the normal AT both in the non-activated and activated states. Based on the "generalized correlation" analysis, the allosteric pathways were also affected. This variant seems to have destabilizing effects in addition to the heparin-binding defect. However, we could not observe significant dissociation of the pentasaccharide in any of the AT-pentasaccharide complex simulations. From the simulation trajectories, we were able to draw qualitative conclusions on the pentasaccharide binding of WT and mutant AT. However, the insufficient conformational sampling of partially or fully dissociated conformations did not allow us to calculate free energies from these data. Such calculations would require a different enhanced sampling technique, for example the recently published LiGaMD method.

5.2. Discussion of results of the nine new mutations

In this study, we present the clinical and molecular characterization of nine novel AT mutations (p.Arg14Lys, p.Cys32Tyr, p.Arg78Gly, p.Met121Arg, p.Leu245Pro, p.Leu270Argfs*14, p.Asn450Ile, p.Gly456delins_Ala_Thr and p.Pro461Thr). Indirect and direct pieces of evidence were collected in order to determine their pathogenic nature and to classify them into ATD subgroups. Among missense variants, the p.Met121Arg, p.Leu245Pro, p.Asn450Ile and p.Pro461Thr mutations were predicted as pathogenic by all six in silico methods (PolyPhen2 HumDiv, PolyPhen2 HumVar, MutPred2, PhD-SNP, SIFT and MutationTaster), and the AT sequence homology study also suggested their deleterious effect.

Two missense mutations, p.Arg14Lys and p.Cys32Tyr are located in the signal peptide sequence of AT. Two of the in silico methods predicted these mutants to be pathogenic, while two other methods designated them as neutral. The sequence homology study was in accordance with this, since the AT signal peptide sequence was heterogeneous in the seven investigated species. The in silico prediction for the p.Arg78Gly mutant was rather conflicting (three methods predicted it as non-pathogenic), however the homology study indicated a strongly conserved position of this mutation.

The p.Arg14Lys mutation resulted in decreased heparin cofactor anti-FXa AT activity (hc-antiFXa), decreased progressive anti-FXa AT activity (p-antiFXa) and decreased AT antigen concentration in the plasma of the patient, suggesting a rather quantitative ATD. The amount of recombinant p.Arg14Lys AT, however (as shown by results of Western blotting, AT antigen and activity measurements) was not significantly lower than that of the WT AT protein. This and the position of this variant (affecting the N-terminal region of positively charged basic residues in the signal peptide, which is a rather neutral part of it) could not confirm its deleterious nature unequivocally and suggest that the index patient might also have other causative factors in the background of his DVT and also in lower plasma AT levels. Corral and colleagues suggested that in cases without causative *SERPINC1* defects mutations in other genes involved in transcriptional control of the gene or in post-translation modifications or mutations in proteins involved in the clearance of AT might affect the plasma level of AT. It might also happen that mutations in the deep promoter or intronic regions of SERPINC1, which are not identified by Sanger sequencing and linked to p.Arg14Lys, are responsible for the lower AT levels. Unfortunately, a large family study could not be executed to further investigate the linkage of this variant to the disease.

The p.Cys32Tyr mutation in the patient resulted in low hc-antiFXa and p-antiFXa AT activity values and a proportionally decreased AT antigen. Western blot analysis of the recombinant p.Cys32Tyr protein detected very low AT protein expression, both in the media and in the cell lysates, and it was confirmed by AT antigen measurements. The RT-qPCR detected a normal mRNA level, suggesting a translational or post-translational defect. The p.Cys32Tyr mutation was located in the conserved polar C-terminal region of the signal peptide containing the cleavage site. In silico analysis of signal peptide predicted an abnormal cleavage site after position 32. The signal peptide plays an important role in translation by ribosomes; the nascent protein is bound via the signal peptide to the signal recognition particle, which guides the complex to the endoplasmic reticulum. Certain positions in the signal peptide are therefore critical from the point of view of signal peptide function. Jochmans et al. described a variant

in a patient with type I ATD, where cysteine was replaced with arginine at the same position, and found it pathogenic. We can conclude from our in silico and biochemical studies that p.Cys32Tyr is a deleterious variant leading to type I ATD.

The p.Arg78Gly mutation affects the heparin-binding region of AT. In the case of the patient, the hc-antiFXa AT activity was decreased, while a normal p-antiFXa AT activity and normal AT antigen were detected, suggesting type II HBS AT deficiency. Western blot analysis and AT antigen measurement of the recombinant p.Arg78Gly mutant showed no alteration, however hc-antiFXa was disproportionally decreased. SPR experiments verified an altered heparin–AT interaction. Bravo-Pérez and colleagues identified a variant in the same position, the p.Arg78Gln mutation, which led to an increase in the low-heparin-affinity AT form in plasma. They classified this variant as type II HBS deficiency, which is in accordance to our findings.

The p.Met121Arg mutation caused proportionally low hc-antiFXa and p-antiFXa AT activity and AT antigen values in the proband, which suggested type I ATD. Western blot analysis of the recombinant p.Met121Arg protein suggested a secretion disorder, as we detected high AT protein expression in the cell lysate but low in the media. In accordance with this, the AT antigen level was very high in the cell lysate, and it was low in the media compared to the AT level in the cell lysate. We investigated the N-linked glycosylation with N-glycosidase F digestion, it was similar to the WT indicating normal glycosylation in the ER. A hypothetical explanation for the very high AT antigen in the cell lysates could be the unfolded protein response (UPR). Abnormal proteins accumulate in the ER, and the ER stress is buffered by the activation of this mechanism. When the capacity of the UPR to sustain proteostasis is overwhelmed, cells enter the canonical apoptosis pathway. In the literature, we found two different mutations in this position, p.Met121Ile and p.Met121Lys; however, no expression studies were carried out in their cases. Our experimental findings support the pathogenic nature of p.Met121Arg mutation.

The p.Leu245Pro mutation was carried by a male patient with proximal DVT. Hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low, suggesting type I ATD. Western blot analysis of the recombinant p.Leu245Pro protein suggested secretion disorder, as we detected high AT protein expression in the cell lysate but low in the media. In accordance with this, the AT antigen level was very high in the cell lysate, and it was low in the media compared to the AT level in the cell lysate. Investigation of the N-linked glycosylation with N-glycosidase F digestion indicated normal glycosylation in the ER. In the literature, we found the AT Murcia (p.Lys241Glu) mutation as a neighboring variant. Certain

missense mutations can cause type I deficiencies through their effects on protein folding because misfolded proteins can be degraded in lysosomes or accumulate inside the endoplasmic reticulum. Because of the similarity to p.Met121Lys, we hypothesized that the UPR mechanism might cause the type I phenotype.

The p.Leu270Argfs*14 mutation was detected in a male patient with 5 episodes of DVT. From the clinical point of view, this case showed the most severe thrombotic phenotype. Hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low, suggesting type I ATD. Western blot analysis and AT antigen measurement of the re-combinant p.Leu270Argfs*14 protein revealed low AT both in the media and in the cell lysates. The RT-qPCR detected a normal RNA level, suggesting a translational defect. After position 270, a stop sequence was generated, causing early termination of translation.

The p.Asn450Ile mutation was carried by a family. Their hc-antiFXa and p-antiFXa AT activity and AT antigen values were proportionally low, suggesting type I ATD. West-ern blot analysis of the recombinant p.Asn450Ile protein showed low AT protein expression for both the media and the cell lysate. We detected an abnormal AT protein with faster electrophoretic mobility on the SDS-PAGE, which suggests an abnormal post-translational modification. Therefore, we investigated the N-linked glycosylation with N-glycosidase F digestion, but it was similar to the WT, indicating normal N-glycosylation in the ER. Defects in other post-translational mechanisms, however, might be responsible for the development of type I ATD.

The p.Gly456delins_Ala-Thr mutation in the proband, which resulted in a protein that was one amino acid longer, led to proportionally low hc-antiFXa and p-antiFXa AT activity and AT antigen values in accordance with a type I ATD. Western blot analysis of the recombinant p.Gly456delins_Ala-Thr protein showed high AT expression in the cell lysates but low in the media. This result suggested a secretion disorder, but not due to abnormal N-linked glycosylation. Jochmans et al. described a sequence change at position 456, which replaced glycine with arginine (p.Gly456Arg) and was classified as pathogenic.

The p.Pro461Thr mutation was detected in three unrelated patients. Hc-antiFXa and p-antiFXa AT activity values were disproportionally low compared to AT antigen values, suggesting a rather functional AT deficiency. Western blot analysis and AT antigen measurement of the recombinant p.Pro461Thr protein detected high AT protein levels both in the media and in the cell lysates. Heparin cofactor activity was decreased compared to progressive activity. SPR measurements verified an altered heparin-AT interaction suggesting

the presence of an IIPE variant. In accordance with our findings, we found the p.Pro461Leu mutation classified as type IIPE and the p.Pro461Ser classified as type IIPE in the literature.

In the case of novel mutations, confirmation or refutation of their pathogenic nature is important in order to help in patient management. Moreover, by introducing the mutations and their consequences into databases, it will help others with future diagnostics. Because the laboratory assays in ATD have limitations and the AT activity measured in plasma is often not in accordance with the clinical phenotype of the patient it is important to estimate the effect of a novel mutation on the structure and function of AT by in vitro experiments. In our present study, severe type I ATD was confirmed for p.Cys32Tyr, p.Leu270Argfs*14 and p.Asn450Ile (altered synthesis) and for p.Met121Arg, p.Leu245Pro and p.Gly456delins_Ala_Thr (altered secretion). All these patients suffered from at least one episode of thrombosis; moreover, multiple thrombosis was registered in most cases. In case of the type II heparin binding site ATD (p.Arg78Gly), which is suggested to be less severe, the patient did not suffer thrombosis and the diagnosis of ATD was established only upon a throughout laboratory investigation before an in vitro fertilization procedure. She was treated with LMWH during her pregnancy, but no long-term anticoagulation was introduced afterwards, and she is still symptom-free. The p.Pro461Thr mutation has a pleiotropic effect on AT, and its clinical severity is considerable; all patients with this mutation suffered thrombosis. However, either provoking factors were explored in the background, or the patient was older at the time of the thrombotic episode. Finally, the pathogenic role of p.Arg14Lys was equivocal according to our investigations. One of the patients carrying this variant is symptom-free, and the other has suffered one thrombotic episode. It cannot be excluded that a major (not registered) provoking factor was also present in the background of his thrombosis or that the decrease in the AT level was caused by a different, currently unidentified genetic defect in the family.

In summary, in our study, we provided insight into the pathogenic nature of different SERPINC1 mutations by in vitro expression studies and in silico analysis, by which different mechanisms of pathogenicity were suggested.

6. SUMMARY OF THE NEW FINDINGS

In this study we examined three, already known, IIHBS type AT mutations (ATBp3, AT Basel, AT Padua) and investigated nine novel AT mutations (p.Arg14Lys, p.Cys32Tyr, p.Arg78Gly, p.Met121Arg, p.Leu245Pro, p.Leu270Argfs*14, p.Asn450Ile, p.Gly456delins_Ala_Thr, p.Pro461Thr) that have not been reported before.

According to our results there may be different molecular mechanisms in the background of altered AT-heparin interaction in case of type IIHBS AT mutations. AT Padua mutation has the strongest effect on AT. We observed the slowest AT-heparin complex formation and the weakest interaction with heparin. AT Padua also exerted a larger low-affinity fraction than normal fraction in CIE experiments. Molecular modeling studies well explain these findings, as AT Padua showed conformational changes in the N-terminal 30-35 region of AT and highly increased fluctuations in RMSF analysis suggesting that the mutation affects both the conformation and the allostery of distant parts of AT Padua molecule. AT Basel binds to heparin more slowly, than other mutants maybe because of the conformational change in the 22-46 loop. Once AT-heparin complex is formed the allosteric activation of AT Basel and the stability of AT Basel-heparin complex might be only slightly affected. ATBp3 showed the fastest and strongest AT-heparin complex formation in SPR studies, however the allosteric activation of ATBp3 is affected, moreover the increased fluctuation in multiple regions of the molecule suggested that this variant has a destabilizing effect. These findings together with the decreased thermostability of homozygous ATBp3 isolated from human plasma suggest the presence of a quantitative component in the pathogenicity of this mutation.

Type I ATD caused by altered protein synthesis (p.Cys32Tyr, p.Leu270Argfs*X14, p.Asn450Ile) or secretion disorder (p.Met121Arg, p.Leu245Pro, p.Gly456delins_Ala_Thr) was proved in six mutants, while type II heparin binding site ATD (p.Arg78Gly) and pleiotropic effect ATD (p.Pro461Thr) were suggested in two mutants. Finally, the pathogenic role of p.Arg14Lys was equivocal. We provided evidence to understand the pathogenic nature of novel SERPINC1 mutations by in vitro expression studies.

7. PUBLICATIONS

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List of publications related to the dissertation

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8. KEYWORDS

Antithrombin, antithrombin deficiency, mutation analysis, thrombosis, genotype-fenotype associations, expression study, in silico methods, molecular modelling, heparin-binding site, surface plasmon resonance

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