

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

**DETECTION AND DIFFERENTIAL DIAGNOSTIC OF HEPARIN BINDING SITE
ANTITHROMBIN DEFECT**

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The Examination takes place at the library of the Department of Pediatrics, Faculty of Medicine, University of Debrecen at 12:00 am, 19th May, 2015.

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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 14:00, 19th May, 2015.

INTRODUCTION AND REVIEW OF LITERATURE

The structure and physiology of antithrombin

Antithrombin (AT) is a single-chain glycoprotein with a molecular mass of 58.2 kDa. The mature protein consists of 432 amino acids with three internal disulfide bonds. AT has two isoforms, which differ only in the extent of glycosylation. The α isoform is N-glycosylated on four Asn residues (95, 135, 155 and 192), while the β isoform lacks glycosylation on Asn135. The α variant is the major AT isoform (90%–95%) in the circulation, the β isoform represents only 5%–10% of AT in plasma. AT is synthesized in the liver, its half-life in the circulation is approximately 2.4 days. A prominent feature of AT is its high affinity binding to negatively charged glycosaminoglycans (GAGs) such as heparin or heparan sulfate which contain specific pentasaccharide units. Due to the lack of carbohydrate residue on Asn135, the β isoform binds to GAGs with higher affinity. Heparan sulfate in the form of heparan sulfate proteoglycane (HSPG) is present on the surface of vascular endothelium. Thus, a higher proportion of the β isoform becomes cleared from the circulation and targets the vessel wall.

AT belongs to the family of serine protease inhibitors (serpins), the largest family of protease inhibitors that consists of over 1500 members. These are single chain globular proteins that consist of 300–500 amino acid residues and show about 30% sequence identity. Serpins share a common tertiary structure; they contain three β -sheets (A-C) and eight to nine α -helices (A-I). A flexible peptide loop, reactive center loop (RCL) containing the reactive site, is exposed on the top of the molecule. RCL contains a sequence, which is complementary to the active site of the target protease. All serpins feature significant structural flexibility, which allows dramatic structural changes upon reaction with the protease to be inhibited. They are so-called suicide inhibitors. The target protease cleaves a scissile bond in RCL and then it remains covalently linked to the inhibitor.

Antithrombin is a misnomer, the inhibitory effect of AT is not restricted to thrombin (factor IIa; FIIa). It is a polyvalent serpin that also inhibits activated factor X (FXa) and to a lesser extent a whole series of serine proteases involved in the hemostatic machinery, including FIXa, FXIa, FXIIa, plasmin and kallikrein. AT inactivates FVIIa only when it is bound to tissue factor. AT is a so-called progressive inhibitor; the rate of its reaction with the active coagulation factors is slow, but in the presence of heparin or HSPG, the rate of inhibition is accelerated 500-fold. AT has a typical serpin secondary and tertiary structure. It consists of nine helices and three β -sheets. In the uncleaved form, it can exist in two main

conformational states. In the native uncleaved form, the 24-membered RCL with the scissile P1-P1' (Arg393-Ser394) bond is outside the main body of AT. In the latent conformation, the RCL is inserted into the β -sheet. The latter conformation is thermodynamically more stable than the native form, which is kinetically "trapped" in a high-energy state. AT circulates primarily in this kinetically trapped native form. The X-ray structure of this conformation revealed that the P1 residue (Arg393) points to the surface of the body of AT, and the P14-P15 residues are inserted into β -sheet A, which constrains the RCL and allows contacts between the P1 arginine side chain and the body of AT. Having such a rigid conformation of RCL, AT is a poor inhibitor of FXa or thrombin, and is unable to inhibit FIXa.

The binding of pentasaccharide or heparin containing the pentasaccharide unit causes remarkable changes in the conformation of RCL and its close proximity. The entrapped part of RCL is expelled from β -sheet A, the end of the third β -strand of β -sheet A moves closer to the fifth β -strand and helix D becomes elongated. The interaction between AT and the pentasaccharide unit takes place in two steps; an initial weak binding intermediate becomes transformed into a high binding state with 1000-fold higher affinity. The latter conformation is necessary for the effective formation of the Michaelis-complex between AT and FXa or FIXa, in which Arg393 of AT and the region in its immediate vicinity is recognized by the protease as a substrate loop. The mechanism of Michaelis-complex formation between thrombin and AT is somewhat different. In this case, the conformational change induced by the allosteric effect of pentasaccharide is not sufficient, and probably not even required. Thrombin also binds to heparin, and the bridging effect of heparin of 18 saccharide units or longer, which brings thrombin and AT together, is essential for effective interaction. After the rate-controlling Michaelis-complex formation, the inhibition of active coagulation factors follows the general scheme of serpin action. In the first step of proteolytic reaction, an acyl-enzyme intermediate is formed through an ester bond between Arg393 and the active site serine of the protease. AT undergoes a rapid, drastic and irreversible conformational change where the P14-P3 part of RCL becomes incorporated into β -sheet A as an additional strand, and AT assumes a cleaved relaxed form. This process is accompanied by a 1000-fold reduction in heparin affinity and by a large-scale conformational change in the acyl-enzyme complex. The protease, which is covalently tethered to Arg393 becomes transported from the top to the bottom of AT, approximately 70 Å away from its original position. Due to the distortion of the active site of the protease, the acyl intermediate becomes stabilized and the second step of proteolytic reaction, the release of the cleaved peptide, cannot take place. In this process the protease structure becomes disrupted and the catalytic triad distorted. Only

very slow release of the inactive inhibitor and enzyme can be detected from the AT-protease complex.

In vivo, the AT-protease complex is rapidly eliminated from the circulation by utilizing a common serpin-protease complex clearance pathway. Binding to members of the low-density lipoprotein receptor family, primarily to low-density lipoprotein-related protein which is an important receptor in the liver, is the main pathway of the elimination of serpin-protease complexes.

The gene for human AT (*SERPINC1*) is located at the 1q23-q25 position. It contains seven exons producing a 1.4 kb messenger RNA (mRNA), and six introns. A leader sequence of 32 amino acids is encoded by exon 1 and the 5' end of exon 2. The heparin binding site of AT is encoded by exon 2 and exon 3a. The reactive site, located in the carboxy-terminal part of the protein, is encoded by exon 6.

The role of antithrombin in the regulation of coagulation

AT serves as a highly important regulator of hemostasis; its absence is incompatible with life. The primary actions of AT are the inhibition of thrombin mediated fibrin clot formation and the generation of thrombin by FXa. As mentioned earlier, AT also inhibits activated clotting factors higher up in the intrinsic (FIXa, FXIa, FXIIa) and extrinsic (FVIIa-tissue factor complex) pathways. It also inhibits a series of other non-coagulant effects of these clotting factors, including platelet activation, vascular cell signaling, proliferation, cytokine production, etc. There are two paradoxes concerning the effect of AT and its importance in the regulation of clotting machinery: 1) it is a weak progressive inhibitor of activated clotting factors, 2) it fails to inhibit effectively fibrin-bound thrombin and FXa present in an activation complex on the platelet surface.

The association of active clotting factors with the surface of activated platelets and with the fibrin clot significantly modifies the inhibitory effect of AT or AT-heparin. When present in the prothrombinase complex on platelets or phospholipid surfaces, FXa escapes the inhibition by AT. Thrombin bound to fibrin or to fibrin degradation products becomes refractive to inhibition by the AT-heparin complex. It is of interest that FVIIa becomes sensitive to inhibition by AT only when bound to tissue factor. The above findings suggest a double role for the AT and AT-HSPG complex in the physiological regulation of blood coagulation. It might control low-level thrombin formation that occurs physiologically in the unperturbed circulation by the inhibition of tissue factor-FVIIa complex, FXa and perhaps other active clotting factors. AT might also exert a scavenger function by neutralizing FXa

and thrombin that have escaped from the clot and from the activation complex. The latter mechanism could prevent the propagation of the clot to areas away from the site of vascular injury.

Epidemiology of antithrombin deficiency

The prevalence of inherited AT deficiency in the general population is estimated to be between 1:2000 and 1:3000. The prevalence of AT deficiency in patients with venous thromboembolism (VTE) is much higher, between 1:20 and 1:200. In a cumulated analysis of 1705 selected patients with VTE, the frequency of AT deficiency was 2.4%. During a mean follow-up time of 2.3 years the incidence of venous thrombosis was high; being 12% in individuals with hereditary AT deficiency in a small Italian cohort. For comparison, the incidence of thrombosis in protein C (PC) and protein S (PS) deficiency was 2.8% and 3.3%, respectively.

In the large prospective EPCOT study (European Prospective Cohort on Thrombophilia), the risk of first VTE in asymptomatic AT, PC or PS deficient individuals and in individuals with Factor V Leiden mutation was analyzed. During the 5.7 year of follow-up, 4.5% of these individuals developed VTE; the annual incidence of first VTE was the highest in those with AT deficiency (1.7%/year). Based on the prevalence data in the general population and in VTE patients, the relative risk of VTE in patients with AT deficiency was estimated to be approximately 25–50-fold.

The risk of VTE conferred by hereditary AT deficiency is the highest among inherited thrombophilias. However, the risk of VTE seems to vary according to the subtypes of AT deficiency. Heterozygous type II HBS AT deficiency (see later) is a less severe phenotype than other heterozygous subtypes.

AT deficiency also represents an increased risk for development of pulmonary embolism (PE) in deep venous thrombosis (DVT), and an increased risk for recurrence of VTE. In an Italian study of patients with proximal DVT, the risk of PE was 2.4-fold in AT deficient patients compared to individuals who developed DVT without inherited thrombophilia. In AT deficiency, the annual incidence of recurrent VTE was found to be 10%, the adjusted hazard ratio for the recurrence of VTE was 1.9.

Molecular genetic background of antithrombin deficiency, genotype-phenotype correlations

The first case of AT deficiency was described by Egeberg in 1965. The first functional AT defect, AT Budapest, was reported by Sas et al. in 1974. According to the recommendations of the International Society on Thrombosis and Haemostasis, AT deficiency is classified as type I (quantitative) and type II (qualitative) deficiency. In type I deficiency, AT activity and the antigen concentration are equally decreased, suggesting defective synthesis or secretion of the protein. In type II deficiency, the defect may involve the reactive site (type II RS), the heparin-binding site (type II HBS) or it can exert a pleiotropic effect (type II PE).

The inheritance of AT deficiency, in general, is autosomal dominant. However, in the case of type II HBS deficiency, it often shows incomplete penetrance or an autosomal recessive pattern. The majority of AT deficient patients are heterozygous for the defect typically with approximately 50% AT activity. Homozygosity is incompatible with life, with the exception of type II HBS variant. The molecular genetic background of AT deficiency is heterogeneous. The mutations are best summarized in the Antithrombin Mutation Database and in the database of human gene mutation data (HGMD).

Almost 50% of the 215 different mutations that have been reported in the HGMD are missense mutations. Small deletions and insertions are also common, contributing 20% and 10%, respectively. Non-sense mutations and splicing site mutations represent 8% and 5% of all reported causative sequence variants, respectively. Whole or partial gene deletions are relatively frequent (5%), while complex rearrangements are rare.

Type I AT deficiencies are most commonly caused by insertions or deletions leading to frameshift and premature stop codon, or less commonly by non-sense mutations. These mutations, which obviously explain the type I phenotype, are primarily the result of unstable mRNA transcripts and/or the presence of truncated proteins. Large gene segment deletions also lead to type I deficiency. Amino acid changes caused by single nucleotide substitutions within the coding region of SERPINC1 may also lead to type I deficiency. In this case, the absence of mutant protein in the circulation is due to mis-folding or a secretion defect.

The type II AT deficiencies are most commonly caused by missense mutations. Among the mutations known to involve the reactive site domain, two regions are preferred: the hinge region (most frequently residues Ala382 and Ala384) and around the reactive-site domain at residues Gly392 (AT Stockholm), Arg393 and Ser394. Most of the missense mutations leading to type II HBS deficiency affect residues Pro41 (AT Basel), Arg47 (AT

Padua I), Leu99 (AT Budapest 3) and Arg129. Practically all patients with AT Budapest 3 (p.Leu99Phe) mutation described to date were of South-Eastern European origin, which may suggest a founder effect. Type II PE deficiency is caused by mutations involving residues 402, 404–407 and 429. This region is responsible for both the structural and functional integrity of AT.

Homozygous type I AT deficiency is not compatible with life and heterozygous patients usually suffer severe thrombosis at a young age. The same stands for type II RS and type II PE deficiencies. However, there is at least one notable exception. The heterozygous p.Ala384Ser mutation (AT Cambridge II) causes type II RS deficiency with a mild phenotype, and this mutation can also exist in homozygous form. Type II HBS deficiency confers a lower risk of thrombosis compared with the other subtypes. Homozygous type II HBS patients usually survive, thrombosis may develop even earlier (frequently in childhood) than in patients with heterozygous type I, or other type II deficiencies.

Symptoms of AT deficiency are DVT and/or PE, which are often recurrent. DVT not infrequently develops at unusual sites, such as in the proximal extremities, and in mesenteric, renal, portal, retinal and cerebral veins. Intracardial atrial thrombosis has also been reported. In addition to venous thrombosis, occasionally, arterial thrombosis has also been described in patients with AT deficiency. The risk of thrombosis conferred by AT deficiency to pregnant women is significantly greater than in other deficiencies. The estimated risk is 1:2.8 for women with type I deficiency, which is approximately 350-times higher than the risk conferred by pregnancy alone.

Acquired antithrombin deficiency

In healthy full-term newborns, the concentration of AT is in the range of 51%–75% of adult average values. Due to severe immaturity of the liver, in preterm infants AT concentrations can be much lower. AT concentrations reach adult ranges by the age of 1 year.

Production of AT is reduced in liver disease with impaired hepatic function. In patients with nephrotic syndrome or other diseases associated with renal or enteral protein loss, the low AT concentration is due to increased elimination. Low concentrations of AT as a result of consumption are found in patients with sepsis, disseminated intravascular coagulation, large thrombus, thrombotic microangiopathy, acute hemolytic transfusion reactions and malignancies.

Long-term therapy with unfractionated heparin is a common cause of moderate AT consumption, which is probably the result of greatly enhanced formation of thrombin-AT

complex in plasma. Therapy with L-asparaginase leads to intracellular retention of AT within the endoplasmic reticulum

Laboratory diagnosis of antithrombin deficiency

A first-line test for the diagnosis of AT deficiency should detect all deficiencies, i.e., AT deficiencies due to decreased AT concentration as well as to a defective molecule. Therefore, the first line test should be a functional assay. The original methods where the inhibition of thrombin by diluted native serum or defibrinated plasma was measured by fibrinogen clotting are impractical and inaccurate and not in use any longer.

With the modern chromogenic (amidolytic) assays, the inhibition of thrombin or FXa activity by AT is measured using thrombin/FXa specific tri-, or tetra-peptide substrates which show sequential similarity to the P1-P3 or P1-P4 sequences of the natural substrates of these enzymes. The peptides conform to the active site of the respective active clotting factor and a para-nitroaniline (pNA) or 5-amino-2-nitrobenzoic acid (ANBA) group is attached to their C-terminal end. Thrombin or FXa rapidly release the pNA or ANBA group from their peptide substrate. Free pNA or ANBA, as opposed to the peptide-bound form, has strong light absorption at 405 nm and its release can be easily monitored spectrophotometrically.

In theory, the assays can be performed in the presence of heparin (heparin cofactor activity) or without heparin (progressive activity). In the former assays, the inhibition of active clotting factors is very quick, while in the latter cases more time is required for AT to exert its inhibitory action. As only the heparin cofactor activity is decreased in all subtypes of AT deficiency, the assay measuring this activity is the generally accepted first line test for the diagnosis of deficiency. Heparin binds to AT making it highly reactive with thrombin or FXa. Thrombin or FXa is added in excess to AT and a part of it becomes rapidly complexed with heparin-AT; in the complex AT activity is abrogated. The extent of thrombin/FXa inhibition depends on plasma AT activity, and the residual free thrombin or FXa is inversely related to AT activity. The amount of free thrombin or FXa is measured using the chromogenic substrates described above. The increase in absorbance at 405 nm can be measured using a kinetic or end-point method, and the change of absorbance is converted to AT activity using a calibration curve. Reference plasma of known AT activity is used to construct the calibration curve. A WHO international standard (2nd International Standard Antithrombin, Plasma, NIBSC code: 93/768) with an assigned potency of 0.85 International Units (IU) is available from the National Institute for Biological Standards and Control (NIBSC; Potters Bar, UK).

This international plasma standard should be used by companies for the calibration of their reference plasma and this information should be stated on the application sheet.

Human thrombin was used in previous thrombin inhibition assays. Human thrombin also reacted with heparin cofactor II and made the assay relatively insensitive for the detection of AT deficiency. In most commercial kits, human thrombin has been replaced by bovine thrombin, which shows minimal reaction with heparin cofactor II. FXa does not react with heparin cofactor II at all. Measurement of AT antigen concentrations is required for the classification of AT deficiencies. Traditional electro-immunodiffusion and radial-immunodiffusion techniques are too time consuming, imprecise; their use is no longer recommended. At present, latex-enhanced immuno-nephelometry is the most frequently used method for measurement of AT antigen concentrations, and commercial kits for this purpose are available.

It is rather surprising that no reference interval determined according to the guideline (C28-A3) from the Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA) is available for AT activity and antigen. A number of different “normal” ranges, varying within a narrow interval have been reported in the literature and are available in manufacturer’s application sheets. Accepting 80% of the average normal (0.8 IU/mL) as the lower limit of reference interval for AT activity seems to be an acceptable compromise, and most laboratories use this value. The upper limit of the reference interval does not have any clinical relevance.

Measurement of AT activity and concentration is not recommended within 3 months of an acute event. During this period, if values are within the reference interval, the exclusion of AT deficiency is possible, but the diagnosis of AT deficiency cannot be confirmed.

In a number of cases, AT determination is requested for patients who are on anticoagulant therapy. Oral anticoagulant therapy with vitamin K antagonists, such as warfarin or acenocoumarol might increase the level of AT, while administration of unfractionated heparin decreases the concentration of AT. Low molecular weight heparins do not have such an effect. For these reasons, we do not recommend diagnosing AT deficiency in patients who are undergoing unfractionated heparin therapy. Also, we do not recommend to exclude AT deficiency during oral anticoagulant therapy. In our experience switching from oral anticoagulant therapy to low molecular heparin for 10 days prior to blood collection is a good compromise that allows the measurement of valid AT values.

THE AIM OF THE STUDY

1. The generally used AT assay is a chromogenic test in which the inhibition of FIIa or FXa by the patient's plasma is measured by a chromogenic peptide substrate. The assays are performed in the presence of heparin; in theory, they should detect, among others, type II HBS deficiencies. However, the effect of heparin on the interaction of AT with FIIa or FXa involves different mechanisms; therefore, the efficiency of AT assays based on the inhibition of the two active clotting factors might be different. The aim of the study was to compare the diagnostic efficiency of anti-FIIa and anti-FXa AT assays in detecting type II HBS AT deficiency.
2. It was assumed that the progressive anti-FXa activity, measured in the absence of heparin, is insensitive to HBS defect and its parallel measurement with hc-anti-FXa activity provides a tool for the diagnosis of type II-HBS deficiency. The aim of study was to develop and evaluate an anti-FXa chromogenic AT assay, which measures both the hc-anti-FXa and p-anti-FXa activity, and to establish their reference intervals.
3. We also intended to test the usefulness of developed assays in the diagnosis of type II HBS deficiency on a relatively high number of AT deficient patients. Based on the results we aimed to develop an algorithm for the diagnosis and classification of AT deficiencies.

MATERIALS AND METHODS

Reference samples

The reference sample group consisted of 188 apparently healthy individuals (104 females and 84 males) older than 18 years of age; median age was 34, interquartile range (IQR): 26 – 41. The health status was checked by questionnaire. Exclusion criteria were acute diseases, any medication (except hypertension treatment), history of arterial and venous thrombosis. Pregnant women and women being on oral contraceptive or estrogen treatment were also excluded.

Clinical samples

Thirty-seven consecutively diagnosed patients with AT deficiency, proved by fluorescent DNA sequencing, were recruited for the study of comparing of hc-anti-FXa and hc-anti-FIIa assays. Among the recruited patients, seven had a type I defect, one had type II PE, and 29 patients from 20 families had type II HBS deficiency (nine carried the mutation in homozygous form, while 20 patients were heterozygotes)

Seventy-eight consecutively diagnosed patients with AT deficiency, confirmed by fluorescent DNA sequencing, were recruited for comparing of hc-anti-FXa and p-anti-FXa assays study. Among the recruited patients, eight had type I defect, one had type II-PE, and 69 patients had type II-HBS deficiency. Among type II-HBS deficient patients 18 carried the mutation in homozygous form, while 51 patients were heterozygotes.

Anti-FXa determinations were also performed on the plasma samples from 24 first-degree relatives of the patients, in which causative AT mutation was excluded by DNA sequencing.

Ethical approval

The study protocol was approved by the Regional and Institutional Ethics Committee of the University of Debrecen and by the Institutional Ethics Committee of Borsod-Abaúj-Zemplén County Teaching Hospital. The work was carried out according to the principles laid down in the Declaration of Helsinki. Written informed consent was obtained from all participants.

Blood collection

Blood samples were collected into Vacutainer tubes containing 0.105 mol/L trisodium citrate (Becton-Dickinson, Franklin Lakes, NJ, USA) after overnight fasting. Plasma was separated by two consecutive centrifugations (1500 g, 15 min, 23 °C). Samples were stored at -70 °C.

Determination of AT hc-anti-FIIa activity

Dade Behring Berichrom antithrombin III test (Marburg, Germany) was used to determine anti-FIIa activity; the reagent kit includes bovine thrombin and tosyl-Gly-Pro-Arg-5-amino-2-nitrobenzoic acid isopropylamide substrate.

Method:

1. 20 µl 6-fold diluted plasma
2. 20 µl physiological saline
3. 180 µl thrombin + heparin
4. 3-min incubation
5. 30 µl substrate
6. measurement at 405 nm, 85 sec

The change of absorbance is converted to AT activity using a calibration curve. The calibration curve for the assay using Standard Human Plasma (Siemens Healthcare, Marburg, Germany) at 0-125% AT activity.

Determination of AT hc-anti-FXa activity

Two assays were used to determine hc-anti-FXa activity. The Siemens (Marburg, Germany) Innovance antithrombin kit (hc-anti-FXa1) uses human FXa and benzoylcarbonyl-D-Leu-Gly-Arg-ANBA-methylamide acetate substrate.

Method:

1. 30 µl Tris-HCl buffer, pH 8.0
2. 10 µl 4-fold diluted plasma
3. 80 µl reagent of FXa (activity is 1.0 U/mL) and heparin (1.5 IU/mL)
4. 3-min incubation
5. 80 µl substrate (2.4 mM)
6. measurement at 405 nm, 60 sec

The $\Delta A/\text{min}$ values were converted to percentage of mean normal anti-FXa activity.

The assay developed by us (hc-anti-FXa2; Antitrombin H+P, Labexpert, Debrecen, Hungary). uses bovine FXa and succinyl-Ile-Glu(γ Pip)Gly-Arg-pNA HCl substrate. Heparin was from Sigma-Aldrich (St Louis, MO, USA).

Method:

1. 10 μ l 10-fold diluted plasma (1 U/mL heparin in the dilution buffer)
2. 50 μ l FXa (12 nkat/mL)
3. 1-min incubation
4. 50 μ l substrate (1.25 mg/mL)
5. measurement at 405 nm for 60 sec

The $\Delta A/\text{min}$ values were converted to percentage of mean normal anti-FXa activity.

For first study, World Health Organization AT reference plasma (National Institute for Biological Standards and Control, Potter Bar, England) was used as a calibrator. For the second study, calibration curve was set-up by measurement on dilutions of HemosIL™ Calibration plasma (Instrumentation Laboratory, Milano, Italy) recalibrated on the mean p-anti-FXa activity and hc-anti-FXa activity in the reference population as 100%. The hc-anti-FXa activity assigned for this calibration plasma by the manufacturer only slightly (+ 2%) deviated from the values calculated on the basis of the means obtained in the reference population.

Determination of p-anti-FXa activity

The following assay protocol for the p-anti-FXa assay was adapted to Siemens BCS coagulometer (Marburg, Germany) and to Ceveron oagulometer (Technoclone, Vienna, Austria). Ten microliter plasma was diluted five-fold by the addition of 40 μ L 50 mmol/L pH 8.4 Tris-HCl buffer containing 175 mmol/L NaCl, 7.5 mol/L EDTA and 10 mg/L polybrene (Sigma-Aldrich, St Louis, MO, USA).

Method:

1. 10 μ l plasma
2. 40 μ l buffer (10 μ g/mL polybrene)
3. 50 μ l FXa (12 nkat/mL)
4. 5-min incubation
5. 50 μ l substrate (1.25 mg/mL)
6. measurement at 405 nm, 60 sec

The $\Delta A/\text{min}$ values were converted to percentage of mean normal anti-FXa activity. HemosIL™ Calibration plasma (Instrumentation Laboratory, Milano, Italy) was used for the construction of calibration curve. The plasma was recalibrated by comparing to the mean p-anti-FXa activity and hc-anti-FXa activity in the reference population. The mean anti-FXa activities were considered as 100%.

Determination of AT antigen

AT antigen was measured by immunonephelometry (Siemens, BN ProSpec® System AT-III).

Method evaluation

Abnormal and Normal Control, (Instrumentation Laboratory) were used for the evaluation of precision performance. The evaluation was carried out according to the EP15-A2 guideline of Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA) using single run per day with duplicate determinations for 20 days.

For the estimation of constant error due to hemoglobin, bilirubin and triglyceride interference plasma samples were supplemented with red blood cell lysate, Intralipid (Baxter, Deerfield Park, IL, USA) and concentrated bilirubin solution in dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA), respectively (CLSI document C56-A).

To determine what portion of p-anti-FXa activity was due to the inhibition of FXa by plasma proteins other than AT, the anti-FXa activities of AT deficient plasma (Enzyme Research Laboratories, Swansea, UK), 2.0 mg/mL purified α_1 -antitrypsin and α_2 -macroglobulin (both from Sigma-Aldrich) were also measured.

Reference intervals were established as described in the CLSI EP28-A3c guideline.

Statistical analysis

GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) 5.0a and SPSS v16a (SPSS Inc. Chicago, IL, USA) were used for statistical analysis. The distribution of the results was analyzed by the Kolmogorov-Smirnov test with Lilliefors correction and by the Shapiro-Wilk tests. Student's t-test was used to analyze differences in variables between the subgroups of individuals.

RESULTS

Comparing of hc-anti-FIIa és hc-anti-FXa AT activity in the plasma of AT-deficient patients

The two anti-FXa AT assays (hc-anti-FXa1: Siemens; hc-anti-FXa2: Labexpert) gave practically identical results with all AT-deficient patients. Anti-FIIa and hc-anti-FXa results in type I deficient patients and in the single type II PE patient did not differ significantly; all were uniformly below 80%, the lower limit of the reference interval. In contrast, with a single exception, anti-FIIa activity of type II HBS heterozygotes was in the reference interval (range: 76%-128%), whereas all hc-anti-FXa activities in this group were below the reference interval (range: 55%-73% for the hc-anti-FXa1 method and 46%-74% for the hc-anti-FXa2 method). In the case of type II HBS homozygotes, all anti-FIIa activities except one were below the reference interval (range, 48%-80%). It is to be noted that in p.Leu99Phe homozygotes, AT antigen levels were in the lowest quartile of the reference interval or below, which suggests that the mutation, in addition to interfering with the binding of heparin, somewhat decreases the AT level. In type II HBS homozygotes hc-anti-FXa activities were much lower than anti-FIIa activities; they were in the ranges of 13% to 25% and 9% to 23% using hc-anti-FXa1 and hc-anti-FXa2 methods, respectively.

Development of chromogenic progressive anti-FXa assay

For the measurement of p-anti-FXa activity the hc-anti-FXa assay had to be modified at several points. Polybrene was included in the assay buffer to neutralize heparin in the plasma of patients treated with heparin or to eliminate the effect of heparin contamination. As in the absence of heparin the inhibition of FXa by AT progresses only slowly, the incubation time and the plasma volume had to be increased to obtain a well-measurable inhibition. For the final routinely used assay, 5-fold diluted plasma and 300 sec incubation time were selected, i.e., the plasma volume was increased 10-fold and the incubation time was prolonged 5-fold as compared to the hc-anti-FXa assay. The calibration curve for the assay using HemosIL™ Calibration plasma.

The evaluation of progressive anti-FXa assay

The assay has an excellent reproducibility, within laboratory imprecision was below 4%.

Hemoglobin up to 500 mg/L, bilirubin up to 200 μ mol/L and triglyceride up to 10 mmol/L did not interfere with the assay.

The hc-anti-FXa activity of the plasma immunodepleted from AT did not differ significantly from zero. In contrast p-anti-FXa activity of this plasma was 20.4% of mean normal p-anti-FXa activity measured on the reference population. The p-anti-FXa activity of 2.0 mg/mL α_1 -antitrypsin corresponded to 9.5% of normal plasma p-anti-FXa activity, while α_2 -macroglobulin in the same concentration exhibited only 4.0% inhibitory activity.

Reference intervals for p- and hc-anti-FXa activities

The distribution of both hc-anti-FXa and p-anti-FXa activities was normal as verified by the Kolmogorov-Smirnov test with Lilliefors correction (hc-anti-FXa d:0.043, p:0.2; p-anti-FXa d:0.058, p:0.2) and by the Shapiro-Wilk tests (hc-anti-FXa d:0.994, p:0.634; p-anti-FXa d:0.989, p:0.154). No outlier was found among the results.

Calculation of the reference interval for hc-anti-FXa activity by parametric and non-parametric method gave practically identical results, 82% – 118% and 81% – 117% of normal average, respectively. For p-anti-FXa activity the calculated reference interval was 82% – 118% (parametric method) and 84% – 117% (non-parametric method).

Comparison of hc-anti-FXa activity, p-anti-FXa activity and AT antigen concentration in patients with antithrombin deficiency

The great majority of the patients had type II HBS deficiency, and among them p.Leu99Phe (AT Budapest 3) was the dominant mutation. Among symptomatic AT deficient patients diagnosed in our laboratory between 2010 and 2013, 81% proved to be type II HBS deficient and 88% of whom possessed the p.Leu99Phe mutation. In addition to the 18 homozygotes and 43 heterozygotes for the p.Leu99Phe mutation, three heterozygous patients with p.Pro41Leu (AT Basel) mutation and five heterozygotes with p.Arg47His (AT Padua I) mutation were also included in the group of patients with type II HBS AT deficiency.

As expected, in type II HBS AT deficient patients with homozygous p.Leu99Phe mutation the hc-anti-FXa activities were very low (mean: 13.1%, median 12%, total range: 8% – 26%). In contrast, the p-anti-FXa activities were much higher, overlapping with the reference interval (mean: 78.4%, median: 77%, total range 64% – 106%). The AT antigen concentrations (mean: 76.4%, median: 79%, total range 51% – 98%) corresponded to the p-anti-FXa activities. The somewhat lower than normal p-anti-FXa activities and AT antigen

concentrations in this group suggest that the mutation, in addition to abrogating heparin binding, to a minor extent, also influences the synthesis/secretion of the molecule.

In p.Leu99Phe heterozygotes the decrease of hc-anti-FXa activity corresponded to the heterozygous state (mean: 50.8%, median: 51%, total range: 34% – 65%), while the p-anti-FXa activity (mean: 90.5%, median 90%, total range: 66% – 111%) only marginally decreased and there was no overlap between the ranges of the two anti-FXa activities. In this group both the mean and median AT antigen concentration were 99% (total range: 78% – 118%). The number of type II HBS AT deficient patients with mutations other than p.Leu99Phe was too small to allow detailed evaluation. However, even in the combined group of patients heterozygous for p.Pro41Leu or p.Arg47His mutation the hc-anti-FXa activities were below the lower limit of reference interval, while the p-anti-FXa activities were within the reference interval and were comparable to the AT antigen values.

In the case of type I heterozygotes and in the single type II PE heterozygote both anti-FXa activities were below the reference interval, just like the AT antigen values in type I deficient patients.

p-anti-FXa/hc-anti FXa ratio in the diagnosis of type II HBS AT deficiency

The above results suggested that comparing p-anti-FXa and hc-anti-FXa activities could be a useful tool in the diagnosis of type II HBS AT deficiency.

The ratios for type II HBS heterozygotes (mean: 1.78, median: 1.74, total range: 1.54-2.21) are well above the upper limit of the reference interval for p/hc ratios (0.87 – 1.14), and are clearly separated from the group of wild type relatives (mean and median: 1.03, total range: 0.92-1.09). In the case of homozygous type II HBS AT deficient patients the ratios are very high (mean: 6.76, median: 6.57, total range: 3.23-9.63), much higher than those of heterozygotes.

In the case of type I AT deficient patients the p-anti-FXa activity is somewhat higher than the hc-anti-FXa activity, which is reflected in ratios being above the reference interval (mean: 1.24, median: 1.21, total range: 1.16-1-35).

Algorithm for the diagnosis and classification of AT deficiency

If hc-anti-FXa AT activity is $\geq 80\%$ AT deficiency is excluded. If the activity is $< 80\%$ to exclude acquired AT deficiency repeated tests are required from different blood samples collected from the same person. If feasible, we recommend a time interval of at least 3 months between the two blood collections. If the hc-anti-FXa AT is repeatedly equal to or $< 70\%$ and

acquired causes had been excluded, the laboratory diagnosis of inherited AT deficiency can be established. Between 70% and 80% of AT activity, it is highly recommended to confirm the diagnosis by molecular genetic testing. Once AT deficiency is diagnosed, the next step is its classification, which occurs in two steps. Plasma AT antigen concentrations are measured to differentiate between type I and type II deficiency. Decreased AT antigen implies type I deficiency, while AT antigen in the normal range indicates type II deficiency. Finally, it is clinically important to distinguish II HBS subtype from other type II variants by performing a progressive activity assay. As opposed to other type II subtypes, HBS variants have normal progressive activity.

DISCUSSION

The clinical phenotype of type II HBS AT deficiency is different from that of type I, and other type II AT deficiencies. Heterozygous type II HBS deficiency represents milder type of thrombophilia the severity of which is comparable to that of heterozygous FV Leiden mutation. In this case homozygosity is not lethal, but severe thrombosis usually occurs at an early age (frequently in childhood). A distinction between type II HBS and other type of AT deficiencies as well as between the homozygous and heterozygous forms is of considerable clinical importance. It is interesting that type II HBS (mainly AT Budapest 3) occurs with high frequency in Hungary.

The first line test in the diagnosis of AT deficiency is a functional chromogenic hc assay, according to our results preferably an anti-FXa assay. The mechanism of Michaelis-complex formation between FIIa and AT is somewhat different from that of FXa and AT. AS it has been mentioned, in the former case, the conformational change of AT induced by the allosteric effect of pentasaccharide is not sufficient and probably not even required. FIIa also binds to heparin, and the bridging effect of heparin consisting of 18 saccharide units or more is essential for the high-affinity interaction between FIIa and AT. For this reason, a mutation in the HBS might have a more profound effect on the hc-anti-FXa than on the anti-FIIa activity of AT in the presence of heparin.

Hc AT activity was determined by measuring the inhibition of FIIa or FXa by the patient's plasma. Differences in the assay conditions would not be predicted to account for different sensitivities between anti-FXa and anti-FIIa assays in detecting a type II HBS defect. The results of this study suggest that anti-FIIa assays cannot detect heterozygous type II HBS AT deficiency and might even miss some homozygotes. In contrast, the hc-anti-FXa activities were diagnostic in all cases. In countries such as Hungary where type II HBS deficiency occurs with high frequency, we recommend the use of an hc-anti-FXa assay as the first-line test. The situation might vary among different geographical areas with regard to first-line functional assay detection of AT deficiency. In the British and Spanish population, the Cambridge II mutation (p.Ala384Ser), which results in moderate thrombosis risk and a moderate decrease in AT activity, is the most prevalent cause of AT deficiency. The anti-FIIa AT activity assay seems to be somewhat more sensitive for detection of the Cambridge II AT defect than the hc-anti-FXa assay, although even with this assay there is an overlap between controls and heterozygotes. The diagnosis of Cambridge II deficiency could be reliably established only by means of molecular genetic methods.

In type II HBS AT deficiency in the absence of heparin binding, AT exerts a progressive anti-FXa and anti-FIIa activity. A chromogenic anti-FXa assay in the absence of heparin has been advocated and used in the diagnosis of a patient with type II HBS AT deficiency. Unfortunately, this assay has not been evaluated. In the application sheet of some of the commercially available hc-anti-FXa assays it is mentioned that the test can be carried out in the absence of heparin, but no specific details are given.

Here we developed an anti-FXa assay, which, with a simple change of buffer, can be used for both p-anti-FXa and hc-anti-FXa activity determinations. The plasma volume was increased 10-fold and the incubation time was prolonged 5-fold as compared to the hc-anti-FXa assay. The inclusion of polybrene in the assay buffer of p-anti-FXa measurement made it possible to perform the assay on plasma from heparin-treated patients and unnoticed heparin contamination would not interfere with the assay. The method is of excellent reproducibility and hemoglobin, bilirubin and triglyceride up to high concentrations do not interfere with the assay. We have adapted it to two routine coagulometers: Siemens BCS (Siemens Healthcare, Marburg, Germany) and Ceveron (Technoclone, Wien, Austria)

To our surprise no reference interval determined according to CLSI guideline (C28-A3) was found in the literature for hc-anti-FXa activity. A number of different 'normal' ranges, varying within a relatively narrow interval, have been reported and are available in manufacturers' application sheets. Based on these results 80% of the average normal (0.8 IU/mL) is generally accepted as the lower limit of reference interval. Following the C28-A3 guideline we found a value of 81% (0.81 U/mL), which confirmed the generally used lower limit.

From clinical point of view it is desirable to distinguish among subtypes of AT deficiencies with different risk of thromboembolic complications. The usefulness of parallel p-anti-FXa and hc-anti-FXa determination proved to be a valuable tool in the classification of AT deficiencies. The range of p/hc ratios for II HBS heterozygous patients (total range: 1.54 – 2.21) was well above the upper limit of the reference interval (total range: 0.87 - 1.14) and clearly separated from that of wild type relatives (total range: 0.92 – 1.09). Most importantly, the p/hc ratios also allowed a clear distinction between type II HBS heterozygotes and homozygotes (range: 3.23 – 9.63); such distinction is of considerable clinical importance. The p/hc ratios for patients with type I AT deficiency were also above the reference interval (total range: 1.16 – 1.35) but below the range of type II HBS heterozygotes. The finding that α_1 -antitrypsin and α_2 -macroglobulin present in the plasma also possess some heparin-independent anti-FXa activity (approximately 20%) explains this result. As the classification

of type I deficiency is based on concomitantly low AT activity and antigen level, even the overlap of the ranges of type I and type II HBS heterozygotes would not cause any diagnostic problem.

In summary, the first line test in the diagnosis of AT deficiency is a functional chromogenic hc assay, preferably an hc-anti-FXa assay. We developed a chromogenic anti-FXa assay that allows the parallel determination of p-anti-FXa and hc-anti-FXa activities. The assay has excellent reproducibility and, even in the absence of molecular genetic investigation, provides a reliable diagnosis of type II HBS deficiency and distinguishes type II HBS homozygotes and heterozygotes.



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List of publications related to the dissertation

1. **Kovács, B.**, Bereczky, Z., Selmeczi, A., Gindele, R., Oláh, Z., Kerényi, A., Boda, Z., Muszbek, L.:
Progressive chromogenic anti-factor Xa assay and its use in the classification of antithrombin deficiencies.
Clin. Chem. Lab. Med. 52 (12), 1797-1806, 2014.
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2. **Kovács, B.**, Bereczky, Z., Oláh, Z., Gindele, R., Kerényi, A., Selmeczi, A., Boda, Z., Muszbek, L.:
The Superiority of Anti-FXa Assay Over Anti-FIIa Assay in Detecting Heparin-Binding Site Antithrombin Deficiency.
Am. J. Clin. Pathol. 140 (5), 675-679, 2013.
DOI: <http://dx.doi.org/10.1309/AJCPVY4Z9XZMFOTH>
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3. Muszbek, L., Bereczky, Z., **Kovács, B.**, Komáromi, I.: Antithrombin deficiency and its laboratory diagnosis.
Clin. Chem. Lab. Med. 48 (Suppl.1), S67-S78, 2010.
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