The Superiority of Anti–FXa Assay Over Anti–FIIa Assay in Detecting Heparin–Binding Site Antithrombin Deficiency

Bettina Kovács, MD,1,2 Zsuzsanna Bereczky, MD, PhD,1 Zsolt Oláh, MD, PhD,3 Réka Gindele, MSc,1 Adrienne Kerényi, MD, PhD,4 Anna Selmeczi, MD,3 Zoltán Boda, MD, PhD,3 and László Muszbek, MD, PhD1,5

From the 1Clinical Research Center, 3Institute of Medicine, Division of Hemostasis, and 4Institute of Laboratory Medicine, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary, 2Central Laboratory, Borsod-A.-Z. County Teaching Hospital, Miskolc, Hungary, and 5Vascular Biology, Thrombosis and Hemostasis Research Group of the Hungarian Academy of Sciences, University of Debrecen, Debrecen, Hungary.

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ABSTRACT [Au1: Structured abstract used per style. Pls review and revise accordingly]

Objectives: Antithrombin is a progressive inhibitor of active factor X (FXa) and thrombin (FIIa). Its effect is 500- to 1,000-fold accelerated by heparin or heparan sulfate. Heterozygous type I (quantitative) and most type II (qualitative) antithrombin deficiencies highly increase the risk of venous thromboembolism (VTE), while homozygous mutations are lethal. The functional defect affecting the heparin-binding site confers moderate risk of VTE to heterozygous and high risk of VTE to homozygous individuals.

Methods: Antithrombin activity assays based on the inhibition of FIIa and FXa were compared for their efficiency in detecting heparin-binding site defects.
**Results:** With a single exception, in heterozygotes for heparin-binding site defect (n = 20), anti-FIIa activities remained in the reference interval, while anti-FXa activities were uniformly decreased. In individuals who were homozygous for heparin-binding site mutation (n = 9), anti-FIIa activities were in the range of 48% to 80%; the range of anti-FXa activities was 9% to 25%. Anti-FIIa and anti-FXa activities in type I deficiencies and type II pleiotropic deficiency did not differ significantly.

**Conclusions:** Anti-FXa antithrombin assay is recommended as a first-line test to detect type II heparin-binding site antithrombin deficiency.

Antithrombin (AT) is a member of the serine protease inhibitor (serpin) family and is a key regulator of the coagulation system. A prominent feature of AT is its high affinity binding to negatively charged glycosaminoglycans, heparin, or heparan sulfate, which contain specific pentasaccharide units. The main physiologic function of AT is to inactivate activated coagulation factor X (FXa) and thrombin (FIIa). It also inhibits other active clotting factors, like FIXa, FXIa, FXIIa, and FVIIa complexed with tissue factor. AT is a progressive inhibitor, the rate of its reaction with the active coagulation factors is slow, but in the presence of heparin or heparan sulfate, the rate of inhibition is accelerated 500- to 1,000-fold. A single pentasaccharide unit is sufficient to transform AT into a high binding state, which is necessary for the effective formation of the Michaelis complex between AT and FXa. Similar to all serpins, AT contains a flexible reactive center loop (RCL) with a scissile bond (Arg393-Ser394) at the C-terminal part. The target protease cleaves this bond and then it becomes covalently captured by the inhibitor. Part of the antithrombin RCL is entrapped and minimally available for interaction with its potential targets. The binding of pentasaccharide or heparin-containing pentasaccharide units to the N-terminal part of the molecule induces the expulsion of RCL from its entrapped position.

AT is an essential thromboprotective molecule; its lack is incompatible with life. Quantitative AT deficiency (type I) and most qualitative (type II) deficiencies exist only in heterozygous form. The cumulative prevalence of AT deficiencies in the general population is estimated to be between 1 in 2,000 and 1 in 5,000, whereas among patients with venous thromboembolism (VTE), its frequency is between 1 in 20 and 1 in 200. AT deficiency also represents an increased risk for the development of pulmonary embolism and for the recurrence of VTE. The risk of VTE conferred by hereditary AT deficiency is the highest among those with inherited thrombophilias; however, it varies considerably according to the subtypes of AT deficiency.
Type I AT deficiencies, most commonly caused by insertions or deletions, occur only in heterozygous form and both AT activity and antigen are around 50%. In type II AT deficiencies, commonly caused by missense mutation, AT activity is decreased, whereas AT antigen level is in the reference interval or the activity-antigen ratio is significantly decreased. There are three subtypes of type II AT deficiency. Mutation may occur in or around the reactive site (type II RS deficiency) or may influence the heparin-binding-site (type II HBS deficiency). Mutations in a region that is responsible for both the structural and functional integrity of AT exert pleiotropic effects (type II PE deficiency).¹⁰

The first-line test for the diagnosis of AT deficiency is a functional assay that should detect both type I and type II deficiencies. 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 amino acid sequences of FIIa and FXa peptide substrates resemble the P1-P4 region of the respective native protein substrate and they are C-terminally linked to a chromogenic group, p-nitroaniline or 5-amino-2 nitro benzoic acid. The chromogenic leaving group, when cleaved off by the enzyme, absorbs strongly in the visible spectral region. The inhibition of the FIIa/FXa-catalyzed release of chromogenic group is the measure of AT activity. The assays are performed in the presence of heparin, ie, 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. This study compared the diagnostic efficiency of anti-IIa and anti-Xa AT assays in detecting type II HBS AT deficiency.

Materials and Methods

Thirty-seven consecutively diagnosed patients with AT deficiency, proven by fluorescent DNA sequencing, were recruited for the study. The study protocol was approved by the National Ethics Committee and informed consent was obtained from all participants. Heparin cofactor AT activity was determined by measuring the inhibition of FIIa or FXa by the patient’s plasma. Dade Behring Berichrom antithrombin III test (Marburg, Germany) was used to determine anti-FIIa activity; the reagent kit includes bovine thrombin and tosyl-Gly-Pro-Arg-5-amino-2-nitrobensoic acid isopropylamide substrate. Anti-FXa activity was performed with two assays: Siemens (Marburg, Germany) Innovance antithrombin kit (anti-FXa1) and Labexpert (Debrecen, Hungary) antithrombin H+P assay (anti-FXa2). The former uses human FXa with benzoylcarbonyl-D-Leu-Gly-Arg-5-amino-2-nitrobensoic acid
methylamide acetate substrate, whereas in the latter kit, FXa is of bovine origin and the substrate is succinyl-Ile-Glu(γPip)Gly-Arg-paranitroaniline HCl. AT antigen was measured with immunonephelometry (BN ProSpec System AT-III, Siemens). For all AT assays WHO AT reference plasma (National Institute for Biological Standards and Control, Potter Bar, England) was used as calibrator. AT determination was carried out at least 3 months apart from the thrombotic events. Among the recruited patients, seven had type I defect, one had type II PE, and 29 patients had type II HBS deficiency. The third group represented 20 families. Among type II HBS-deficient patients nine carried the mutation in homozygous form, while 20 patients were heterozygotes Table 1.

Results

The two anti-FXa AT assays gave practically identical results with all AT-deficient patients (Table 1, Figure 1). Anti-FIIa and anti-FXa results in type I–deficient patients and in the single type II PE patient (not shown in Figure 1) did not differ significantly; all were uniformly below 80%, the lower limit of reference interval. In contrast, with a single exception, anti-FIIa activity of type II HBS heterozygotes was in the reference interval (range, 76%-128%), whereas all anti-FXa activities in this group were below the reference interval (range, 55%-73% for the anti-FXa1 method and 46%-74% for the anti-FXa2 method). 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. Anti-FXa activities were much lower than anti-FIIa activities; they were in the ranges of 13% to 25% and 9% to 23% using anti-FXa1 and anti-FXa2 methods, respectively. Figure 1 demonstrates that a clear distinction can be made between heterozygotes and homozygotes using anti-FXa assays. The mean anti-FIIa-to-anti-FXa1 ratio was 4.02 (range, 2.96-5.31) for homozygotes, 1.54 (range, 1.24-1.83) for heterozygotes, and 1.16 (range, 0.93-1.34) for the combined type I and type II PE group. Using the anti-FXa2 assay, the respective mean ratios were similar: 4.79 (range, 2.56-6.9), 1.59 (range, 1.19-2.17), and 1.18 (range, 0.93-1.38).

Discussion

The mechanism of Michaelis complex formation between FIIa and AT is somewhat different from that of FXa and AT. 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.\textsuperscript{11-13} For this reason, a mutation in the HBS might have a more profound effect on the anti-FXa than on the anti-FIIa activity of AT in the presence of heparin. In type II HBS AT deficiency, in the absence of heparin binding, AT exerts a progressive anti-FXa and anti-FIIa activity. However, a much higher concentration of AT, that is, much less diluted plasma, and longer incubation time would have been needed to detect such activity.\textsuperscript{14} The plasma is highly diluted in all assays (50- to 100-fold) and the incubation of diluted plasma with FXa in the Siemens anti-FXa assay was 180 seconds, similar to the incubation time with FIIa in the Siemens anti-FIIa assay. The incubation time in the Labexpert anti-FXa assay was even shorter (60 sec). Thus, differences in the assay conditions would not be predicted to account for different sensitivities between anti-FXa and anti-FIIa assays in detecting type II HBS defect.

Heterozygous type II HBS deficiency confers a lower risk of thrombosis compared with the other subtypes.\textsuperscript{15,16} Homozygous patients with type II HBS usually survive, but may develop thrombosis even earlier (frequently in childhood) than patients with heterozygous type I or other type II deficiencies. To detect this type of AT deficiency and to distinguish it from other AT deficiencies is of clinical importance. The results of this study suggest that anti-FIIa assays cannot detect heterozygous type II HBS AT deficiency and might even miss some homozygotes. For this reason, in countries such as Hungary, where type II HBS deficiency occurs with high frequency (although the frequency is not yet known for most other countries), we recommend the use of anti-FXa assay as the first-line test.

We found only a single publication on the occurrence of type II HBS deficiency in the general population; it claims a frequency of 1 in 2,500 to 1 in 3,200 for this defect,\textsuperscript{14} but no supporting data are provided. To our knowledge, no large-scale study has been reported on the percentage of type II HBS deficiency among AT-deficient patients. The high percentage of type II HBS deficiency among our patients, and the predominant occurrence of p.Leu99Phe mutation originally described as antithrombin III Budapest 3,\textsuperscript{17} very likely reflects the general situation in Hungary. Among symptomatic AT-deficient patients diagnosed in our laboratory during the last 4 years (n = 110), 81% (89/110) proved to be type II HBS deficient, 88% of whom (78/89) possessed the p.Leu99Phe mutation (unpublished data, 2013 \textbf{[Au2: Pls provide date of unpublished findings]}). Haplotype analysis of four unrelated kindreds with p.Leu99Phe mutation suggested a founder effect.\textsuperscript{18} An ongoing large-scale population study
in our laboratory seeks to establish the prevalence of this mutation in the Hungarian population and in the population of neighboring countries.

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.\textsuperscript{4,19} Anti-FIIa AT activity assay seems to be more sensitive for the detection of Cambridge II AT defect than anti-FXa assay, although even with this assay there is an overlap between controls and heterozygotes.\textsuperscript{19,20} The diagnosis of Cambridge II deficiency could be reliably established only by means of molecular genetic methods.

Twelve type II HBS AT mutations have been reported in the AT mutation database (http://www1.imperial.ac.uk/departmentofmedicine/divisions/experimentalmedicine/haematology/coag/antithrombin/). A limitation of the present study is the inclusion of a limited number of the known type II HBS AT mutations. Another limitation is the inclusion of only one anti-FIIa assay in comparison studies. Studies on patients with other type II HBS mutations and with more anti-FIIa reagent kits could further strengthen the conclusion drawn from this study.

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Address reprint requests to Dr Muszbek: Clinical Research Center, University of Debrecen, Medical and Health Science Center, 98 Nagyerdei Krt 4032 Debrecen, Hungary; muszbek@med.unideb.hu.

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References


