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Progressive chromogenic anti-factor Xa assay and its use in the classification of antithrombin deficiencies

Abstract

Background: Antithrombin (AT) is a slow-acting progressive inhibitor of activated clotting factors, particularly thrombin and activated factor X (FXa). However, the presence of heparin or heparan sulfate accelerates its effect by several magnitudes. AT deficiency, a severe thrombophilia, is classified as type I (quantitative) and type II (qualitative) deficiency. In the latter case mutations may influence the reactive site, the heparin binding-site (HBS) and exert pleiotropic effect. Heterozygous type II-HBS deficiency is a less severe thrombophilia than other heterozygous subtypes. However, as opposed to other subtypes, it also exists in homozygous form which represents a very high risk of venous thromboembolism.
Methods: A modified anti-FXa chromogenic AT assay was developed which determines both the progressive (p) and the heparin cofactor (hc) activities, in parallel. The method was evaluated and reference intervals were established. The usefulness of the assay in detecting type II-HBS AT deficiency was tested on 78 AT deficient patients including 51 type II-HBS heterozygotes and 18 homozygotes.
Results: Both p-anti-FXa and hc-anti-FXa assays showed excellent reproducibility and were not influenced by high concentrations of triglyceride, bilirubin and hemoglobin. Reference intervals for p-anti-FXa and hc-anti-FXa AT activities were 84%–117% and 81%–117%, respectively. Type II-HBS deficient patients demonstrated low (heterozygotes) or very low (homozygotes) hc-anti-FXa activity with normal or slightly decreased p-anti-FXa activity. The p/hc ratio clearly distinguished wild type controls, type II-HBS heterozygotes and homozygotes.
Conclusions: Concomitant determination of p-anti-FXa and hc-anti-FXa activities provides a reliable, clinically important diagnosis of type II-HBS AT deficiency and distinguishes between homozygotes and heterozygotes.

Keywords: anti-factor Xa assay; antithrombin; antithrombin deficiency; heparin binding-site; thrombophilia.

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Introduction

Antithrombin (AT), a key regulator of the coagulation system, belongs to the family of serine protease inhibitors (serpins) [1–4]. AT shares the common structural/functional feature of serpins; it contains three β-sheets, nine α-helices and a flexible reactive center loop (RCL) with the reactive site, exposed on top of the molecule. The term antithrombin is misleading, in addition to thrombin it also inhibits activated factor X (FXa) and, to a lesser extent, other active serine protease clotting factors, FIXa, FXIa, FXIIa [2, 5] and FVIIa complexed with tissue factor [6–8]. AT has two isoforms that differ only in the extent of glycosylation [2, 5]. The α isoform, predominant in the circulation, is N-glycosylated on Asn residues 95, 135, 155 and 192, while the β isoform lacks glycosylation on Asn135.

AT is a suicide inhibitor, the target protease cleaves the scissile Arg393-Ser394 bond in RCL, and then it remains covalently linked to the serpin. It is a so-called progressive inhibitor, the rate of its reaction with thrombin or FXa is slow. However, the high affinity binding of AT to negatively charged glycosaminoglycans with specific pentasaccharide units (heparin, or heparan sulfate)

results in a 500- to 1000-fold acceleration of the rate of inhibition. The heparin-binding site (HBS) is located in the N-terminal part of the molecule and involves amino acid residues Arg47, Lys114, Lys125, Arg129 [9]. In the absence of heparin P14-P15 residues of RCL are inserted into β-sheet A, which constrains the RCL and makes it minimally available for interaction with its 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. The mechanism by which heparin accelerates the inhibition of thrombin and FXa is different. The allosteric effect of a single pentasaccharide unit is sufficient to transform AT into a high binding state required for the effective formation of Michaelis complex between AT and FXa. The mechanism of heparin-induced effective Michaelis complex formation between thrombin and AT is somewhat different. In this case the conformation change induced by the allosteric effect of pentasaccharide is not sufficient, and might not even be required. Thrombin also binds to heparin and the bridging effect of heparin containing at least 18 saccharide units is essential for its effective interaction with AT [10–12].

AT is a highly important thromboprotective molecule; the lack of AT is incompatible with life. The prevalence of its deficiencies in the general population is between 1 in 2000 and 1 in 5000 [13, 14], whereas the frequency of AT deficiencies among patients with venous thromboembolism (VTE) is between 1 in 20 and 1 in 200 [15, 16]. AT deficiency is classified as type I (quantitative) and type II (qualitative) [17]. In type I deficiency AT activity and the antigen concentration are equally decreased due to defective synthesis or secretion of the protein. Type II deficiency is usually caused by missense mutation involving the reactive site (RS), the HBS, or exerting a pleiotropic effect (PE) [18]. The clinical phenotype of type II-HBS AT deficiency differs from that of other subtypes. As opposed to other subtypes, its homozygous form is not lethal, although it results in severe VTE at an early age. Heterozygous type II-HBS AT deficiency is a less severe phenotype than other heterozygous subtypes. This subtype often shows incomplete penetrance or might even demonstrate autosomal recessive pattern. The frequency of type II-HBS AT deficiency seems to show considerable variation among countries/regions. The single publication on the occurrence of type II-HBS deficiency in the general population claimed a frequency of 1 in 2500 to 1 in 3200 for this defect [19], but no supporting data have been provided. In Hungary this subtype seems to be the most prevalent AT defect; among symptomatic AT-deficient patients diagnosed in our laboratory during the last 4 years (n=110), 81% proved to be type II-HBS deficient.

A scheme on the stepwise diagnosis and classification of AT deficiency has been recommended [3]. The first step in the diagnostic algorithm is a chromogenic screening test measuring the inhibition of thrombin or FXa in the presence of heparin. A recent survey (2013/4) of ECAT Foundation showed that approximately 58% of the laboratories used heparin cofactor (hc) AT assay based on thrombin (FIIa) inhibition while 42% of them used anti-FXa assay. We have reported that the hc-anti-FXa (hc-anti-FXa) assay is superior over the anti-FIIa assay in detecting type II-HBS AT deficiency [20]. However, this screening test in itself does not ensure the diagnosis. It was assumed that the progressive anti-FXa (p-anti-FXa) activity, measured in the absence of heparin, is insensitive to HBS defect and its parallel measurement with hc-anti-FXa activity provides a tool for the diagnosis of type II-HBS deficiency. In this study we modified the anti-FXa chromogenic AT assay to measure both the hc-anti-FXa and p-anti-FXa activity, evaluated the assays, established their reference intervals and on a relatively high number of AT deficient patients demonstrated its usefulness in the diagnosis of type II-HBS deficiency.

Materials and methods

Subjects

Seventy-eight consecutively diagnosed patients with AT deficiency, confirmed by fluorescent DNA sequencing, were recruited for the study. Among the recruited patients, eight had type I defect, one had type II-PE, and 69 patients had type II-HBS deficiency. The latter group represented 51 families. 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. The reference sample group consisted of 188 apparently healthy individuals, 104 females and 84 males, median age: 34 [interquartile range (IQR): 26–41]. Exclusion criteria for the healthy group were the history of arterial and venous thrombosis and, in the case of women, being on oral contraceptive or pregnancy.

Ethics

The work was carried out according to the principles laid down in the Declaration of Helsinki and amended in 2008 by the World Medical Assembly in Seoul, Korea. The study protocol was approved by the National Ethics Committee and informed consent was obtained from all participants.

Determination of heparin cofactor and progressive anti-FXa activity and antithrombin antigen

The following assay protocol for the p-anti-FXa assay was adapted to Siemens BCS coagulometer (Marburg, Germany) and to Ceveron coagulometer (Technoclone, Vienna, Austria). Ten microliter plasma was diluted five-fold by the addition of 40 µL 50 mmol/L pH 8.4 Tris-HCl buffer containing 175 mmol/L NaCl, 7.5 mol/L EDTA and 10 mg/L polybrene. The diluted plasma was incubated with 50 µL 12 nkat/mL FXa for 300 s at 37 °C. Then, 50 µL 1.25 mg/mL BIOPHEN CS-11(32) [Suc-IIe-Gly-(γPip)Gly-Arg-pNA, HCl] chromogenic substrate (Hyphen BioMed, Neuville sur Oise, France) was added and the release of pNA by uninhibited FXa was recorded at 405 nm for 60 s. The same assay components were used for the determination of hc activity, with the following exceptions: in this case polybrene was replaced by 1 U/mL heparin in the dilution buffer, the dilution of plasma samples was 50-fold and the incubation with FXa was shortened to 60 s. The ΔA/min values were converted to percentage of mean normal anti-FXa activity. 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. AT antigen was measured by immunonephelometry (Siemens, BN ProSpec® System AT-III). The antibody used in the assay is a polyclonal one raised against purified AT. It is very likely directed against different epitopes, which are not given by the manufacturer. Anti-FXa activity and AT antigen determinations for patients with thrombosis were carried out at least 3 months from the thrombotic events.

Method evaluation

Commercially available plasmas, HemosIL™ Normal Control and Low Abnormal Control, (Instrumentation Laboratory) were used for the evaluation of precision performance of anti-FXa assays. 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 FXa inhibitor plasma proteins other than AT, the anti-FXa activities of AT deficient plasma (Enzyme Research Laboratories, Swansea, UK), 2.0 mg/mL purified α_1-antitrypsin and α_2-macroglobulin (both from Sigma-Aldrich) were also measured.

Reference intervals were established as described in the CLSI EP28-A3c guideline [21]. The need for partitioning of reference values according to gender was investigated by the *z* statistic and by the ratio of SD values.

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. Calculations and construction of graphs were performed by GraphPad Prism 5.0a software (La Jolla, CA, USA).

Results

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. Figure 1A demonstrates the dependence of anti-FXa activity on plasma dilution and the time of FXa incubation with diluted plasmas. For the final routinely used assay, 5-fold diluted plasma and 5 min 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 is shown on Figure 1B.

The evaluation of progressive anti-FXa assay

The assay has an excellent reproducibility, within laboratory imprecision was below 4% (Table 1). Hemoglobin up to 500 mg/L, bilirubin up to 200 µmol/L and triglyceride up to 10 mmol/L did not interfere with the assay (Figure 2). As in addition to AT, there are other proteins with anti-FXa activity in the plasma, we estimated the contribution of such proteins to the measured p-anti-FXa activity and hc-anti-FXa activity. First, the anti-FXa activities of commercially available pooled plasma immunodepleted from AT was measured. The p-anti-FXa activity of this plasma was 20.4% of mean normal p-anti-FXa activity measured on the reference population. The hc-anti-FXa activity of the immunodepleted plasma did not differ significantly from zero. As α_1-antitrypsin and α_2-macroglobulin also possess some heparin-independent anti-FXa activity [22, 23], they were suspected to be the primary sources of non-AT p-anti-FXa activity and the inhibitory effect of purified α_1-antitrypsin and α_2-macroglobulin in the range

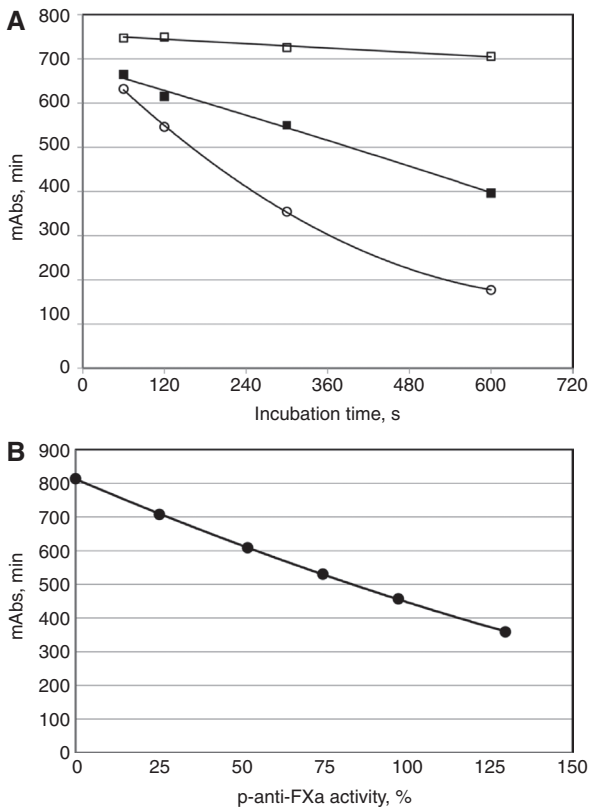

Figure 1 Characteristics of chromogenic progressive (p) anti-FXa activity assay.
(A) The dependence of anti-FXa activity on the dilution of pooled normal plasma and on the time of incubation of FXa with diluted plasmas. The measurements were carried out in the presence of the heparin antagonist, polybrene. □, Fifty-fold plasma dilution; ■, 10-fold plasma dilution; ○, 5-fold plasma dilution. The latter plasma dilution and 300 s incubation time were selected for the routine assay. (B) Calibration curve for the assay. mAbs, milliabsorbance at 405 nm.

of their normal plasma concentration was determined. The p-anti-FXa activity of 2.0 mg/mL α_1-antitrypsin corresponded to 9.5% of normal plasma p-anti-FXa activity, while α_2-macroglobulin in the same concentration exhibited only 4.0% inhibitory activity. These findings indicate that AT is responsible for approximately 80% of p-anti-FXa activity present in the plasma.

Table 1 The precision of progressive anti-FXa assay.

	Normal control plasma	Pathologic control plasma
Mean±s_r	102.3±2.2%	41.9±0.8%
	CV: 2.15%	CV: 1.89%
Mean±s_l	102.3±3.3%	41.9±1.6%
	CV: 3.20%	CV: 3.75%

CV, coefficient of variation; s_l, within-laboratory precision; s_r, repeatability (within-run precision).

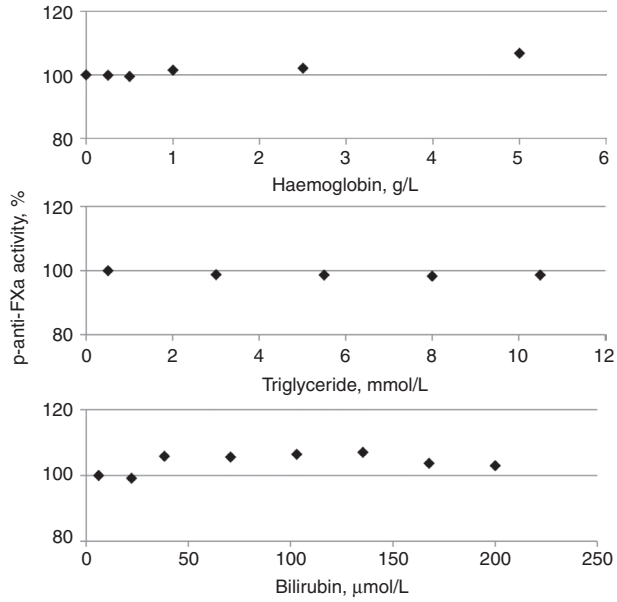

Figure 2 The influence of interfering substances (hemoglobin, triglyceride and bilirubin) on the progressive anti-FXa assay.

Reference intervals for progressive and heparin cofactor anti-FXa activities

The distribution of both hc-anti-FXa and p-anti-FXa activities was normal as verified by two statistical methods (Figure 3). 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 heparin cofactor anti-FXa activity, progressive anti-FXa activity and antithrombin antigen concentration in patients with antithrombin deficiency

Table 2 demonstrates the hc-anti-FXa activity, p-anti-FXa activity and the AT antigen concentration of all individual AT deficient patients. The causative mutations leading to AT deficiency are also shown in the table. The great majority of the patients had type II-HBS deficiency, and among them p.Leu99Phe (AT Budapest 3 [24]) 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 [20]. In addition to the

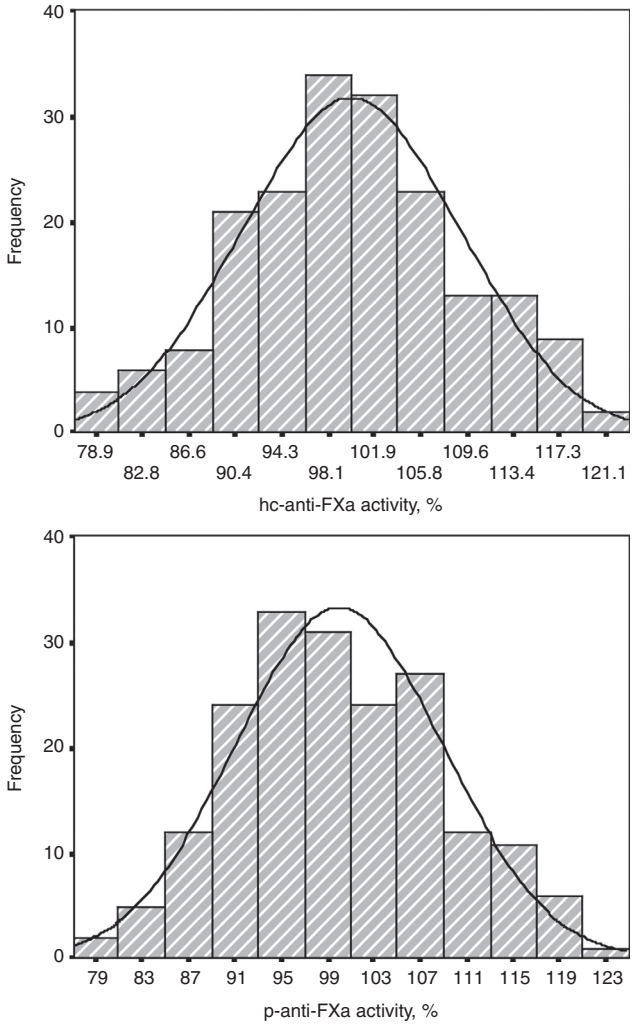

Figure 3 The distribution of heparin cofactor (hc) and progressive (p) anti-FXa activity in a healthy reference population.
The normal distribution of the results was proven for both assays by the Kolmogorov-Smirnov test with Lilliefors correction (hc-anti-FXa d: 0.043, p: 0.2; p-anti-FXa statistic: 0.058, p: 0.2) and by the Shapiro-Wilk test (hc-anti-FXa assay d: 0.994, p: 0.634; p-anti-FXa assay: statistic: d: 0.989, p: 0.154).

18 homozygotes and 43 heterozygotes for the p.Leu99Phe mutation, three heterozygous patients with p.Pro41Leu (AT Basel [25, 26]) mutation and five heterozygotes with p.Arg47His (AT Padua I [27]) 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%) (Table 2). 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%) (Table 2), 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 plus 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 (Table 2).

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 calculated ratios p/hc ratios for each AT deficient group and their healthy relatives are shown on Figure 4. The ratios for type II-HBS heterozygotes 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. In the case of homozygous type II-HBS AT deficient patients the ratios are very high, 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 (Table 2), which is reflected in ratios being above the reference interval (Figure 4). This is very likely due to the approximately 20% contribution of FXa inhibitors other than AT to the plasma p-anti-FXa activity (see above); evidently, the concentration of these inhibitors is not decreased in type I deficiency.

Discussion

Large scale prospective epidemiological studies involving AT, protein C or protein S deficient individuals and

Table 2 Heparin cofactor and progressive anti-FXa activities and antithrombin antigen concentrations in patients with antithrombin deficiency.

AT deficiency	Gender	hc-anti-FXa activity, %	p-anti-FXa activity, %	AT antigen, %	Mutation
Type II-HBS homozygotes n=18	M	8	64	51	p.Leu99Phe
	F	8	77	76	p.Leu99Phe
	F	12	77	80	p.Leu99Phe
	F	16	83	79	p.Leu99Phe
	F	15	88	79	p.Leu99Phe
	F	9	83	74	p.Leu99Phe
	M	20	79	70	p.Leu99Phe
	M	14	106	85	p.Leu99Phe
	F	12	75	77	p.Leu99Phe
	F	10	72	83	p.Leu99Phe
	F	10	66	52	p.Leu99Phe
	M	14	81	90	p.Leu99Phe
	M	10	72	82	p.Leu99Phe
	F	10	66	63	p.Leu99Phe
	F	20	97	84	p.Leu99Phe
	M	8	74	82	p.Leu99Phe
	F	26	84	71	p.Leu99Phe
	F	13	68	98	p.Leu99Phe
Type II-HBS heterozygotes n=51	F	46	94	101	p.Leu99Phe
	F	47	97	99	p.Leu99Phe
	M	58	96	96	p.Leu99Phe
	M	65	101	109	p.Leu99Phe
	F	52	90	90	p.Leu99Phe
	M	54	83	nd	p.Leu99Phe
	M	51	88	97	p.Leu99Phe
	M	41	84	79	p.Leu99Phe
	M	53	93	106	p.Leu99Phe
	F	52	94	88	p.Leu99Phe
	F	50	97	90	p.Leu99Phe
	M	55	102	nd	p.Leu99Phe
	M	54	94	nd	p.Leu99Phe
	M	58	111	100	p.Leu99Phe
	F	53	98	96	p.Leu99Phe
	M	59	102	nd	p.Leu99Phe
	F	48	83	108	p.Leu99Phe
	M	56	87	106	p.Leu99Phe
	M	56	96	118	p.Leu99Phe
	F	51	85	114	p.Leu99Phe
	M	49	98	nd	p.Leu99Phe
	F	57	109	102	p.Leu99Phe
	F	59	107	nd	p.Leu99Phe
	F	63	106	nd	p.Leu99Phe
	F	46	77	92	p.Leu99Phe
	M	52	91	103	p.Leu99Phe
	F	52	89	100	p.Leu99Phe
	M	47	85	96	p.Leu99Phe
	F	51	84	92	p.Leu99Phe
	M	40	80	107	p.Leu99Phe
	F	48	85	106	p.Leu99Phe
	M	34	75	85	p.Leu99Phe
	M	53	86	98	p.Leu99Phe
	F	47	85	102	p.Leu99Phe
	F	49	92	104	p.Leu99Phe
	M	47	84	96	p.Leu99Phe
	M	52	90	102	p.Leu99Phe

(**Table2** Continued)

AT deficiency	Gender	hc-anti-FXa activity, %	p-anti-FXa activity, %	AT antigen, %	Mutation
	F	54	101	108	p.Leu99Phe
	M	50	82	113	p.Leu99Phe
	M	51	86	95	p.Leu99Phe
	F	39	66	78	p.Leu99Phe
	M	48	83	91	p.Leu99Phe
	F	36	74	96	p.Leu99Phe
	M	73	117	108	p.Pro41Leu
	F	54	110	132	p.Arg47His
	F	48	99	120	p.Arg47His
	F	66	126	137	p.Arg47His
	M	66	123	130	p.Arg47His
	F	65	108	124	p.Pro41Leu
	F	74	116	130	p.Pro41Leu
	F	54	86	nd	p.Arg47His
		4			
Type II-PE heterozygote	M	76	78	88	p. Pro407Thr
Type I heterozygotes	F	56	75	58	Arg132X
n=8	F	53	74	54	Arg132X
	F	43	56	56	Arg132X
	M	49	64	63	c.807delT p.Leu238ArgfsX13
	F	46	69	72	p.Glu237Lys
	M	46	61	61	IVS5-14G>A
	F	56	76	64	dup c. 315–319
	M	48	71	52	dup c. 315–319

With the exception of the novel dup c. 315–319 defect, all mutations are included in the antithrombin mutation database [38]. It is to be noted that p.Glu237L mutation (AT Truro [39]) in the database is considered as type II mutation and increased heparin affinity of the recombinant mutant was demonstrated [40, 41]. However, in the original publication the AT antigen and activity levels were 66% and 68%, respectively. As in our study the AT antigen level was also decreased, this deficiency was treated as type I. AT, antithrombin; F, female; FXa, activated factor X; HBS, heparin binding-site defect; hc-anti FXa activity, heparin cofactor anti-FXa activity; M, male; p-anti FXa activity, progressive anti-FXa activity; PE, mutation with pleitropic effect.

individuals with Factor V Leiden mutation indicated that the risk of first VTE conferred by hereditary AT deficiency is the highest among inherited thrombophilias [28]. The relative risk of VTE in patients with AT deficiency was estimated to be approximately 25–50-fold [29–32]. AT deficiency also represents an increased risk of pulmonary embolism and recurrence of VTE [16, 33, 34]. The clinical phenotype of type II-HBS AT deficiency is different from that of type I, and other type II AT deficiencies [19, 35, 36]. 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 might influence the prediction of the risk of recurrent thrombotic events and might also influence the clinical decision concerning the duration of anticoagulant therapy.

The first line test in the diagnosis of AT deficiency is a functional chromogenic hc assay, according to our former results [20] preferably an anti-FXa assay. In the previous study we showed that in 95% of heterozygotes with genetically proven type II-HBS AT deficiency, anti-FIIa activities were in the reference interval, i.e., this test failed to recognize the defect. At the same time the hc-anti-FXa activities were diagnostic in all cases. In homozygotes for type II-HBS AT deficiency the anti-FIIa activity was much higher (48%–80%) than the anti-FXa activity (9%–25%). The addition of AT antigen measurement allows differentiation between type I and type II AT deficiencies, but does not identify the type II-HBS subtype. The diagnosis of this deficiency by functional tests requires the comparison of p-anti-FXa and hc-anti-FXa AT activities, in which tests AT activity is measured in the absence and presence of heparin, respectively. The progressive assay is very much under-utilized in the diagnosis and classification of AT deficiencies [19]. The old clotting assays of AT activity in the absence of heparin were too cumbersome

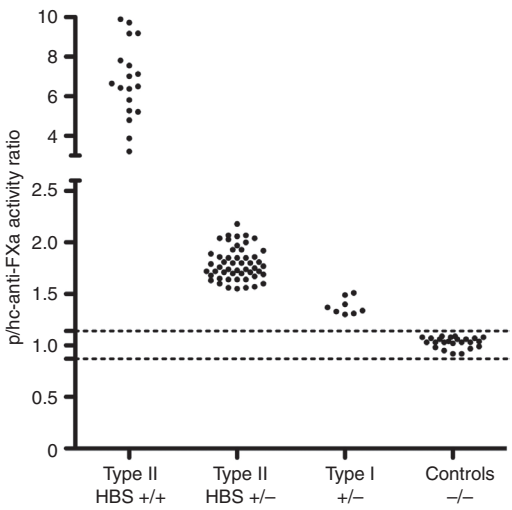

Figure 4 The ratio of progressive (p) and heparin cofactor (hc) anti-FXa activity in different types of antithrombin deficiency and in non-deficient wild type relatives (controls).
HBS, heparin binding-site deficiency; +/+, homozygote; +/–, heterozygote; –/–, wild type controls. The two broken horizontal lines represent the lower (0.87) and upper (1.14) limits of the reference interval for p/hc-anti-FXa ratios. The reference interval was calculated from the results of reference sample group by non-parametric method.

and difficult to interpret [37, 38]. 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 [19]. 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. A former application sheet from HYPHEN BioMed (West Chester, OH, USA) recommended 1-h incubation of FXa with the patient plasma (http://www.aniara.com/pdf/SS-ANIARA-Prog-ATIII.pdf), which hardly allows an automated kinetic procedure. Here we describe 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 5-min incubation time allows automation of the assay; we have adapted it to two routine coagulometers. 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.

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. The same stands for the upper limit, but to our knowledge, no clinical relevance has been associated with AT activities exceeding the upper limit of the reference interval. For the first time we also determined the reference interval for the p-anti-FXa AT assay. Similar to the case of hc-anti-FXa assay, a narrow reference interval (84%–117%) was established for this assay, as well.

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 in the classification of AT deficiencies is demonstrated in Table 2 and Figure 4. The range of p/hc ratios for II-HBS heterozygous patients (1.54–2.21) was well above the upper limit of the reference interval (1.14) and clearly separated from that of wild type relatives (range: 0.92–1.09). The p/hc ratios for patients with type I AT deficiency were also above the reference interval but below the range of type II-HBS heterozygotes. 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 problems. 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. In summary, the chromogenic anti-FXa assay described above allows the parallel determination of p-anti-FXa and hc-anti-FXa activities and, even in the absence of molecular genetic investigation, provides a reliable diagnosis of type II-HBS deficiency and distinguishes between homozygotes and heterozygotes.

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