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Clinical and laboratory characteristics of antithrombin deficiencies; a large cohort study from a single diagnostic center

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Abstract

Introduction: Inherited antithrombin (AT) deficiency is a heterogeneous disease. Due to low prevalence, only a few studies are available concerning genotype-phenotype associations. The aim was to describe the clinical, laboratory and genetic characteristics of AT deficiency in a large cohort including children and to add further laboratory data on the different sensitivity of functional AT assays.

Patients and methods: Non-related AT deficient patients (n=156) and their family members (total n=246) were recruited. Clinical and laboratory data were collected, the mutation spectrum of *SERPINC1* was described. Three different AT functional assays were explored.

Results: Thirty-one *SERPINC1* mutations including 11 novel ones and high mutation detection rate (98%) were detected. Heparin binding site deficiency (type IIHBS) was the most frequent (75.6%) including AT Budapest3 (ATBp3), AT Padua I and AT Basel (86%, 9% and 4% of type IIHBS, respectively). Clinical and laboratory phenotypes of IIHBS were heterogeneous and dependent on the specific mutation. Arterial thrombosis and pregnancy complications were the most frequent in AT Basel and AT Padua I, respectively. Median age at the time of thrombosis was the lowest in ATBp3 homozygotes. The functional assay with high heparin concentration and pH 7.4 as assay conditions had low (44%) sensitivity for ATBp3 and it was insensitive for AT Basel and Padua I.

Conclusion: Type IIHBS deficiencies behave differently in clinical and laboratory phenotypes from each other and from other AT deficiencies. Heparin concentration and pH seem to be the key factors influencing the sensitivity of AT functional assays to IIHBS.

Keywords: antithrombin deficiency, mutation spectrum, genotype-phenotype association, antithrombin activity, assay sensitivity

1. Introduction

Thrombosis is highly frequent in the developed countries and considered as major determinant of morbidity and mortality [1]. Antithrombin (AT) belongs to the serpin superfamily and regulates coagulation by inhibiting thrombin, activated factor X (FXa), and to a lesser extent FIXa, FXIa, FXIIa and FVIIa [2, 3]. Hereditary AT deficiency is classified as type I (quantitative) and type II (qualitative) [4]. In type II deficiency, the defect may affect the reactive site (IIRS), the heparin-binding site (IIHBS) or it can exert pleiotropic effect (IIPE). In general, the complete absence of AT is incompatible with life; in most cases (except for IIHBS) heterozygosity is associated with severe thrombotic symptoms. In IIHBS deficiency homozygosity may be compatible with life, however, those patients suffer from severe thrombosis. The thrombotic risk of heterozygote IIHBS patients is suggested to be less severe than other heterozygotes [5, 6]. In addition to venous thrombosis (VTE), occasionally, arterial thrombotic events (ATE) were also described in AT deficiency [7-12].

The human AT gene (*SERPINC1*) is localized on chromosome 1q23-25, consists of 7 exons and 6 introns and encodes 464 amino acids [5]. The mutation profile of *SERPINC1* is heterogeneous, more than 310 different mutations have been reported (http://www.hgmd.cf.ac.uk; last accessed at August 28, 2017). Missense mutations are the most frequent, but nonsense and splice-site mutations, insertions and deletions may also occur. Genetic analysis is a useful diagnostic tool for confirming inherited AT deficiencies, especially for patients with borderline AT activity values [13]. The mutation detection rate in *SERPINC1* in patients with decreased AT activity is variable among different populations; in general it is 69-83% [13-16]. AT Cambridge II (p.Ala416Ser, IIRS), AT Budapest 3 (ATBp3,

p.Leu131Phe, IIHBS) and AT Basel (p.Pro73Leu, IIHBS) are the most prevalent variants, which are considered as founder mutations [10, 13, 17-21].

For laboratory diagnosis of AT deficiency the first-line test is a chromogenic functional assay, in which the inhibition of thrombin (FIIa) or FXa in the presence of heparin (heparin cofactor activity, hc-anti-FIIa or hc-anti-FXa) is measured [2]. Measurement of AT activity in the absence of heparin (progressive AT activity, p-anti-FXa) is useful for discriminating between IIHBS and other types [22]. The sensitivity of functional assays is various [10, 12, 23]. FXa-based assays are thought to be less sensitive for certain variants around the reactive site, like AT Cambridge II [17, 18]. In contrast, it was published that hc-anti-FXa assays had higher sensitivity to type IIHBS AT deficiency, than hc-anti-IIa assays [12, 24, 25]. An algorithm for laboratory diagnosis of AT deficiency was established, which is based on the determination of hc-anti-FXa, p-anti-FXa, AT antigen and molecular genetic testing [26].

Due to its low prevalence, so far only a few data have been published concerning the clinical and laboratory characteristics of AT deficiency [10, 12-16, 21, 27].

The aim of the present study was to describe and compare the clinical and laboratory characteristics of different types of AT deficiency in a large cohort of patients including a large group of children; to explore the genetic background, to describe novel mutations, to define the mutation detection rate in patients with decreased AT levels in a tertiary diagnostic center and to add further laboratory data on the sensitivity of functional AT assays.

2. Patients and Methods

2.1. Patients

Patients with thromboembolic history and/or pregnancy complications (please see later in details) and reduced hc-anti-FXa AT activity determined from at least two independent blood samples were selected for our study. Thrombosis was described according to guidance of the International Society of Thrombosis and Haemostasis [28]. Briefly, presence of provoking factors was considered if trauma, surgery, hospitalization due to acute illness, central venous catheters, immobilization, pregnancy, oral anticoncipient, hormonal treatment, prolonged travel, L-Asparaginase treatment occurred within one month before the diagnosis of VTE. The presence of acquired risk factors (i.e. chronic situations as malignancy, paroxysmal nocturnal hemoglobinuria, autoimmune diseases, antiphospholipid syndrome, obesity, as BMI above 30kg/m^2, varicose veins, nephrotic syndrome, heart failure, long-term immobilization) was registered. Between January 2007 and August 2016, a total of 156 non-related AT deficient patients (index patients) and their family members (total n=246) were diagnosed at our center.

All enrolled individuals were informed about the study according to the study protocol, and gave written informed consent. Ethical approval for the study was obtained from the National Ethical Council (3166/2012/HER).

2.2. Laboratory methods

Fasting blood samples were collected into 0.109 mol/L citrate vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ, USA) at least three months after the acute thrombotic episode and stored at -80°C until determination. Inherited thrombophilia (protein C and S deficiencies, APC resistance, dysfibrinogenaemia) was investigated

by routine methods on BCS-XP coagulometer (Siemens, Marburg, Germany). Presence of Factor V Leiden mutation (FVL) and prothrombin 20210G>A (FIIG20210A) polymorphism was investigated with LightCycler480 (Roche Diagnostics GmbH, Mannheim, Germany) by using real-time PCR and melting curve analysis.

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

2.3. Molecular genetic analysis of SERPINC1

Genomic DNA was isolated from peripheral whole blood by using the QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany). Sanger sequencing was executed to identify mutations in the exons, flanking intronic regions and in the promoter using an ABI3130 Genetic Analyzer and Sequencing Analysis 5.4 software (Thermo Fisher Scientific, Carlsbad, CA, USA) [29]. If Sanger sequencing did not find causative mutations, multiplex ligation-dependent probe amplification (MLPA)

was performed using SALSA MLPA KIT P227 (MRC-Holland, Amsterdam, the Netherlands). The MLPA products were analyzed by GeneMapper Software 4.1 (Thermo Fisher Scientific).

2.4. In silico prediction of novel missense mutations

The pathogenicity of the novel mutations were evaluated by three prediction methods, PolyPhen2 [30] (http://genetics.bwh.harvard.edu/pph2/index.shtml), MutPred [31, 32], (http://mutpred.mutdb.org), and PhD-SNP [33] (http://snps.biofold.org/snps-and-go/snps-and-go.html) [34]. The PolyPhen2 server offers two (HumDiv and HumVar) scoring schemes, which differ in the training sets on which they were developed [30]. For all the applied methods the score values between 0.5-1.0 mean possibly/probably pathogenic mutations, while score value less than 0.5 predicts likely benign mutations.

2.5. Statistical methods

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

3. Results

3.1. SERPINC1 mutations detected in Antithrombin deficiency

We identified 31 different *SERPINC1* mutations (n=22 type I and n=9 type II) with high mutation detection rate (98%) (Fig. 1). Among the 31 mutations 11 novel ones were detected (36%). Most of the index patients carried type IIHBS mutations (75.6%) due to the high frequency of the founder ATBp3 (86% out of type IIHBS). ATBp3 was registered in homozygous and heterozygous forms, while all other *SERPINC1* mutations were detected only in heterozygous state. AT Basel and AT Padua I were detected in 5 and in 11 families, respectively. Each type IIRS mutation (AT Stockholm, AT Glasgow and p.Ile386Thr) was registered in one family. AT Torino and AT Budapest 5 (type IIPE) were registered in one family and in five families, respectively. Type I deficiency, regardless of the mutation was detected in 25 families.

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

3.2. Genotype-phenotype correlations in patients with SERPINC1 mutations

Clinical characteristics of AT deficient patients according to different types are summarized in Table 1. Detailed description of patients with type I and type II deficiencies are shown in Supplementary Table 1 for known and Table 2 for novel mutations. Except for type IIRS, where all patients were females, there was no significant difference in gender among the different AT deficiency types (Table 1).

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

The time to the first manifestation of VTE and of any thrombotic event (including VTE, ATE and pregnancy complications) was compared between type I, type IIHBS heterozygotes and ATBp3 homozygotes (Fig. 2A and B). When VTE was considered as clinical outcome, the age was significantly lower in type I patients as compared to type IIHBS heterozygotes (p=0.002), while it was significantly higher as compared to ATBp3 homozygotes (p<0.001). When the clinical outcome was considered as a composite of VTE, ATE and pregnancy complications, the time to the first manifestation did not differ significantly between type I and type IIHBS heterozygotes (p=0.064). ATBp3 homozygosity was more severe than type I (p<0.001) in this aspect. Moreover, the frequency of proximal thrombosis was higher in ATBp3 homozygosity as compared to type I deficiency (62.5% vs. 14.3%, p=0.002), while there was no difference between type I and type IIHBS heterozygotes.

There was no significant difference in the ratio of patients with FVL or FIIG20210A mutations among the different AT deficient groups and no other

inherited thrombophilia was registered in any of our patients (Table 1). There were no differences in the ratio of patients with acquired risk factors or provoking factors. Antiphospholipid syndrome was not found in our patients. The number of patients with congenital vascular anomaly was low in all groups.

Due to the high number of patients with type IIHBS deficiency we were able to compare the clinical characteristics among the different subgroups (Table 1). As it was expected, the ratio of symptomatic patients was the highest (92.3%) in ATBp3 homozygotes; it was significantly higher than in ATBp3 heterozygotes ($p=0.001$) and in AT Padua I ($p=0.006$). VTE was more frequent in patients with ATBp3 mutation as compared to AT Padua I and AT Basel (AT Padua I vs. ATBp3 heterozygotes $p=0.041$; AT Padua I vs. ATBp3 homozygotes $p<0.001$; AT Basel vs. ATBp3 homozygotes $p<0.001$). The frequency of VTE was the highest in ATBp3 homozygotes (ATBp3 heterozygotes vs. ATBp3 homozygotes $p<0.001$). ATE was the most frequent in AT Basel (AT Basel vs. ATBp3 heterozygotes $p=0.046$; AT Basel vs. ATBp3 homozygotes $p=0.006$). Pregnancy complications were the most frequent in AT Padua I; however, the difference was not statistically significant among the different type IIHBS groups. The number of patients with proximal localization of thrombus was significantly higher in ATBp3 homozygotes than in ATBp3 heterozygotes ($p=0.001$). There was no significant difference in the presentation of additional thrombosis risk factors among the different type IIHBS groups.

The time to the first VTE was not different between AT Basel and AT Padua I ($p=0.982$) and between AT Basel and ATBp3 heterozygotes ($p=0.095$) (Fig. 2C and D). VTE occurred significantly earlier in ATBp3 heterozygotes than in AT Padua I ($p=0.010$). ATBp3 homozygotes were the youngest at the time of the first VTE (ATBp3 homozygotes vs. AT Basel $p=0.002$; ATBp3 homozygotes vs. AT Padua I

and vs. ATBp3 heterozygotes p<0.001). When the composite outcome was compared, the time to the first manifestation did not differ significantly between AT Basel and AT Padua I (p=0.459), between ATBp3 heterozygotes and AT Basel (p=0.997) and between ATBp3 heterozygotes and AT Padua I (p=0.130). The time to the first manifestation was significantly lower in ATBp3 homozygotes as compared to other type IIHBS groups (ATBp3 homozygosity vs. ATBp3 heterozygosity p<0.001, ATBp3 homozygosity vs. AT Padua I p<0.001 and ATBp3 homozygosity vs. AT Basel p=0.018).

3.3. Pediatric patients with AT deficiency

In our cohort 32 patients had the first thrombotic episode in childhood (i.e. before the age of 18) (Supplementary Table 2). Most of them were of type IIHBS mutants (n=25) and the majority of children (n=18) were homozygous carriers of ATBp3. There were two peaks of age (0-1 years and 12-18 years) at the time of first thrombotic episode.

All but one infants (i.e. 0-1 years, n=7) with thrombosis were ATBp3 homozygotes. Only one of them was a heterozygous carrier of FVL and hereditary vascular anomaly was diagnosed only in 2 children (v. cava inferior aplasia and hypoplasia of both iliacal veins and hypoplasia of v. cava inferior and v. iliaca communis). The infant with AT Truro had sinus sagittal thrombosis and a cerebral vein hypoplasia was explored.

There were 20 patients older than 12 years of age at the time of first thrombotic episode and among them n=15 were carriers of ATBp3 and provoking factor could be explored only in 4 patients. No additional hereditary or acquired thrombosis risk factors were found in the background. One patient with AT Basel

suffered from ischemic stroke and MI and 2 patients with ATBp3 heterozygosity had ischemic stroke.

3.4. Laboratory phenotype of patients with SERPINC1 mutations

Hc-anti-FXa AT activity and p-anti-FXa activity were low and correlated well in type I deficient patients. AT antigen concentrations were below the lower limit of the reference interval in all cases (Supplementary Table 1A, Table 2).

Both hc-anti-FXa activity and p-anti-FXa AT activities were low in all IIRS and IIPE patients and AT antigen levels were all normal (Supplementary Table 1B). It is interesting, that in type IIHBS patients AT levels were different according to the specific mutations. In theory, hc-anti-FXa activity is low in type IIHBS deficiency, while p-anti-FXa activity is normal and the high p-anti-FXa/hc-anti-FXa AT activity ratio discriminates well between type IIHBS and other type II AT deficiencies [2, 22]. In our study the hc-anti-FXa assay that was used for diagnosis, gave low AT activity in all type IIHBS cases (100% sensitivity). In the case of AT Basel and AT Padua I the p-anti-FXa activity and AT antigen were within the reference interval in all patients (as expected); the median of p-anti-FXa/hc-anti-FXa ratio was 1.66; range 1.53-2.05 and it was 1.89; range 1.71-2.10, respectively (Supplementary Table 1B). However, low p-anti-FXa activity and AT antigen concentration were detected in some patients with ATBp3 (especially homozygotes). The median p-anti-FXa/hc-anti-FXa ratio values in heterozygotes and homozygotes were 1.51 (range 1.28-2.11) and 5.60 (range 2.88-8.85), respectively.

The functional assay used for diagnosis was compared to 2 similar (i.e. FXa-based in the presence of heparin) commercially available assays (Table 3). The assays gave similar results in type IIRS and type IIPE patients. In type IIHBS AT Padua I

and Basel AT activity values differed markedly between assay 1 and 2. While assay 1 and our assay gave low AT activity in all cases, assay 2 did not recognize these mutants showing normal AT activity for all patients. AT activity values were decreased in ATBp3 homozygotes with all assays; however, the results obtained by assay 2 were significantly higher than those in the other tests, suggesting heterozygous state. The sensitivity of Assay 2 for ATBp3 heterozygosity was only 44%.

As it was suggested by our findings and by earlier studies [10, 12], the substrate type (i.e. anti-FIIa or anti-FXa assay) is not the only factor that influences the sensitivity of functional AT assays. Here we investigated the effect of heparin concentration and pH of assay conditions on assay sensitivity in a pilot experiment using our diagnostic assay (Fig. 3). Upon increasing the heparin concentration in the assay all AT activity values increased and in one sample with AT Padua I it reached the lower limit of the reference interval. AT activity values further increased, reaching or exceeding the lower limit of the reference interval in 2 AT Basel and in 1 AT Padua I samples by changing the assay conditions to pH 7.4. When the heparin concentration of the original assay increased by 8-fold all AT Basel and Padua I samples gave normal AT activity results. AT activity values of ATBp3 samples did not increase further.

3.5. In silico prediction of novel missense mutations

Sequential alignment for AT in human and in different species showed that all the novel missense mutations affected conservative regions (Fig. 4). The results of the in silico prediction models are summarized in Table 4. The ATBp3 mutation (p.Leu131Phe) and the AT Cambridge II (p.Ala416Ser) mutation were used as

positive controls, since their pathogenicity had been confirmed by different in vitro studies before. All but the p.Leu131Phe mutations were predicted as definitely pathogenic by the methods. Among the novel missense variants the p.Leu205Pro mutation has been already characterized by our group [35, 36].

4. Discussion

In our geographic region 31 different *SERPINC1* mutations were found and among them 11 were novel. In our cohort type IIHBS AT deficiency (especially ATBp3) was the most frequent. The high mutation detection rate (98%) that is observed in our study may be caused mainly by the high prevalence of the founder ATBp3. In the background of lower mutation detection rates in patients with low AT levels an aberrant N-glycosylation was hypothesized, or mutations in the regulatory sequences of *SERPINC1* (eg. c.1-171C>G) were suggested [37-39]. Among our novel AT variants all mutations except for p.Pro461Thr showed a laboratory phenotype of type I deficiency. AT activity and antigen concentrations were around 50% in these heterozygous individuals, indicating that the mutant AT may not appear in the circulation. The p.Pro461Thr mutation is suggested as a type IIPE variant based on our laboratory results and on a publication, in which the p.Pro461Leu was described [40].

Concerning the clinical consequences of AT mutations type I deficiency in general was associated with a severe venous thrombotic phenotype in our cohort, except for AT Wobble, in which case no VTE but MI was registered. AT Truro was considered as type IIHBS variant based on an in silico experiment [41], however, the laboratory phenotype observed in our study and by others [42] suggests a quantitative defect. The biochemical consequences of this mutation therefore would be interesting

to study. According to our knowledge, this paper collected one of the highest number of pediatric AT deficient symptomatic patients to date, where the frequency of type IIHBS was the most prevalent. These findings highlight the importance of AT deficiency screening in unprovoked pediatric thrombosis cases especially in populations, where the prevalence of type IIHBS AT deficiency is high. To date no clear recommendations have been published concerning the therapeutic issues (e.g. the duration of anticoagulation) in AT deficient patients and no sufficient data exist regarding pediatric cases to release a guideline. In our cohort most of the pediatric patients have been put on long-term anticoagulation, however, the risk of bleeding in this special age group must also be considered.

Type IIHBS is an interesting group of AT deficiency, where the clinical and laboratory phenotypes are more heterogeneous and dependent on the specific mutation. ATBp3, AT Padua I and AT Basel associated with not only VTE but also ATE and pregnancy complications were also registered with different frequencies [27, 43, 44]. When the time to the first manifestation of VTE is considered as a measure of clinical severity in our study, it is to be highlighted that type I AT deficiency is more severe than heterozygous IIHBS deficiency, however ATBp3 homozygosity is more severe than type I AT deficiency in this aspect. Within type IIHBS group ATBp3 homozygosity is the most severe and ATBp3 heterozygosity is significantly more severe than AT Padua I. AT Basel and AT Padua I are not significantly different concerning the time to the first manifestation of VTE. When the time to first manifestation of a composite clinical endpoint including not only VTE but also ATE and pregnancy complications is taken into consideration, the severity of type I and type IIHBS heterozygotes do not differ significantly. ATBp3 homozygosity is more severe than type I and type IIHBS heterozygous AT deficiency in this aspect. AT

Basel is rather associated with ATE; the highest number of pregnancy complications is detected in AT Padua I. It is interesting, why no homozygous patients with AT Basel and Padua I were found in our study, however, by screening the literature no such patients had been described elsewhere. It is very likely, that at least one reason for not finding homozygous AT Basel or Padua I patients in our study is the relative rarity of these mutations comparing to the frequency of ATBp3. It is also possible, that – due to their severity - AT Basel and Padua I are lethal mutants in homozygous form.

In our cohort n=29 and n=64 asymptomatic carriers were registered upon family screening below the age of 20 and 50, respectively. Since AT deficiency confers the highest absolute VTE risk among inherited thrombophilia, as demonstrated by prospective studies, the proband's family is worthy of screening for causative mutations [45]. It was suggested that the most beneficial consequence of family-screening was to decrease the incidence of provoked VTE in affected individuals by thromboprophylaxis in high-risk situations and to avoid oral contraceptive prescription [46].

It is interesting that there are big differences in the sensitivity even among hc-anti-FXa assays to type IIHBS AT deficiency. Until recently only a few publications have addressed this problem [10, 12, 21, 22, 23, 26]. It is to be noted, that type IIHBS AT deficiency may be under-diagnosed by some commercially available functional assays. In our present study the assay that was used for diagnosis was able to recognize all AT deficiency types. This assay contains bovine FXa as substrate, BIOPHEN CS-11 [Suc-IIe-Gly-(γ Pip)Gly-Arg-pNA, HCl] as chromogenic substrate, uses a final dilution of 1:150 of test plasma and contains 1000 U/L heparin in a Tris-HCl buffer pH 8.4. Assay1 that was used for comparison was 100% sensitive to all

AT deficiency types including IIHBS. Similarly to Orlando's findings, assay2 had lower sensitivity to ATBp3 and was absolutely insensitive for AT Basel and Padua I [12]. Based on the results of our pilot experiment, it is suggested that the high heparin concentration and perhaps the lower ionic strength are major factors that decrease the sensitivity of the assays to type IIHBS deficiency, especially to AT Basel and Padua I. Our results strengthen the hypothesis that type IIHBS deficiency is a heterogeneous group with different strength of heparin binding according to the specific mutations. This results in different behavior in the functional assays. Assay modifications, that were tested, had less effect on AT activity values of ATBp3 samples, which suggest more complex consequence of this mutation than being a heparin-binding defect.

5. Conclusion

In conclusion, in our large group of AT deficient patients it was possible to investigate the mutation detection rate, the genotype-phenotype associations and to explore the behavior of different types of AT deficiency using three different functional assays. Since AT deficiency belongs to the group of rare diseases it is hardly possible to conduct large clinical studies concerning this disease. In our tertiary hemostasis center by a collection of AT deficient patients during a 10-year period we have described 11 novel causative mutations and described the highest *SERPINC1* mutation detection rate. From the point of view of clinical manifestations type I AT deficiency is rather homogeneous, while type IIHBS seems to be a heterogeneous group. Here we highlight that type IIHBS deficiencies behave differently in clinical and laboratory phenotype from each other and from other AT deficiencies. An international registry of AT deficiency would potentially help in introduction of clinical and laboratory guidelines.

Authorship

RG, GB and IK performed the laboratory experiments; AS, ZO, PI, GP, EM, LN, AN, HL, GM, MK, AS, KR and ZB recruited the patients, collected samples and provided clinical data; LM, GP and AS edited the paper; ZB designed the research; RG and ZB analyzed the results and wrote the paper. All authors read and approved the final manuscript.

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Conflict of interest

The authors state that they have no conflict of interest.

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Legend to figures

Fig. 1. Localization of identified *SERPINC1* mutations in our AT deficient population.

Nucleotide numbering is given to the first nucleic acid of the initiator ATG codon. Mutations are described according to Human Genome Variation Society guidelines. Novel mutations are indicated in Bold and Italic style.

Fig. 2. Comparison of time to the first manifestation of venous thrombosis (A and C) and to the first manifestation of any thrombotic event (B and D) in type I, type IIHBS heterozygotes and in ATBp3 homozygotes.

Kaplan-Meier survival curves illustrate the difference among type I (black solid line, n=47), type IIHBS heterozygotes (dotted line, n=98) and ATBp3 homozygotes (grey solid line, n=26) for venous thrombosis (A) and for any thrombotic event (B) including venous and arterial thrombosis and pregnancy complications. The differences in time to the first clinical event among the different type IIHBS groups for venous thrombosis (C) and for any thrombotic event (D) are also demonstrated. AT Basel (n=7) is represented with a dotted line, AT Padua I (n=15) is represented with a black solid line, ATBp3 heterozygotes (n=76) are represented with a grey solid line and ATBp3 homozygotes (n=26) are represented with a semi-dotted line. Cumulative ratio on the y-axis represents the ratio of individuals without clinical event. Censored cases are marked with circles. The median values of the time at the first clinical event (years) with the 95% Confidence Intervals in the brackets are shown in the figures.

HeZ, heterozygote; HoZ, homozygote

Fig. 3. Effect of assay modifications on the AT activity values of AT Budapest 3 (A), AT Basel (B) and AT Padua I (C) heterozygote samples.

The effect of increased heparin concentration and lowered pH on AT activity values by modifying the original diagnostic assay is shown. The upper limit of the reference interval (80%) is highlighted with thick horizontal line.

Fig. 4. Sequential alignment for AT in human and in different species.

The two sets of short peptide sequence around the site of three novel missense *SERPINC1* mutations are shown. Arrows indicate the location of mutations.

Fig. 1

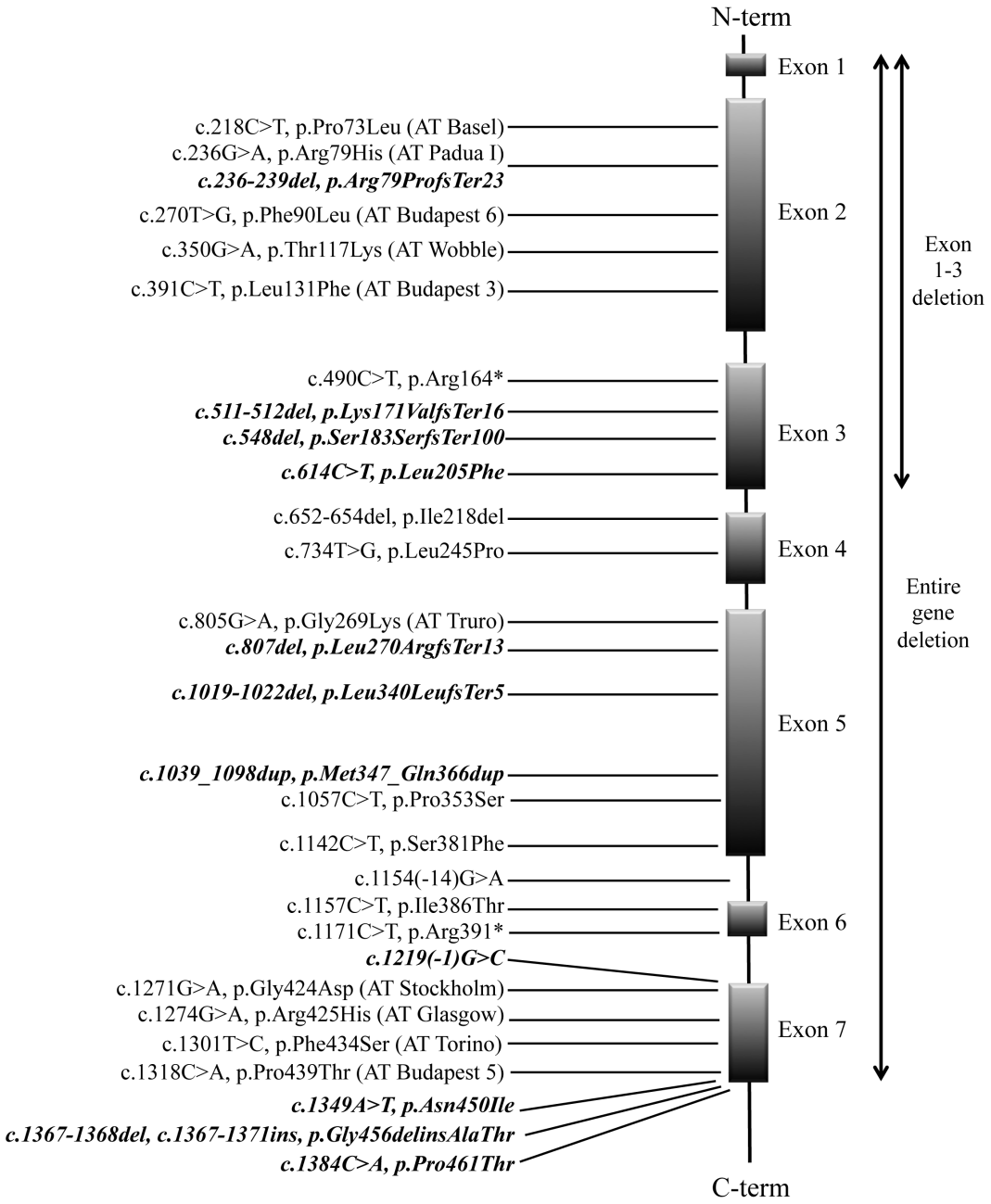
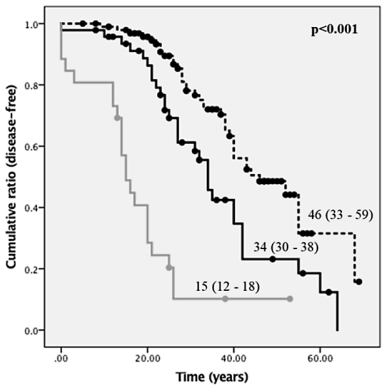

N-term
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
Exon 7
C-term
Exon 1-3 deletion
Entire gene deletion
c.218C>T, p.Pro73Leu (AT Basel)
c.236G>A, p.Arg79His (AT Padua I)
c.236-239del, p.Arg79ProfsTer23
c.270T>G, p.Phe90Leu (AT Budapest 6)
c.350G>A, p.Thr117Lys (AT Wobble)
c.391C>T, p.Leu131Phe (AT Budapest 3)
c.490C>T, p.Arg164*
c.511-512del, p.Lys171ValfsTer16
c.548del, p.Ser183SerfsTer100
c.614C>T, p.Leu205Phe
c.652-654del, p.Ile218del
c.734T>G, p.Leu245Pro
c.805G>A, p.Gly269Lys (AT Truro)
c.807del, p.Leu270ArgfsTer13
c.1019-1022del, p.Leu340LeufsTer5
c.1039_1098dup, p.Met347_Gln366dup
c.1057C>T, p.Pro353Ser
c.1142C>T, p.Ser381Phe
c.1154(-14)G>A
c.1157C>T, p.Ile386Thr
c.1171C>T, p.Arg391*
c.1219(-1)G>C
c.1271G>A, p.Gly424Asp (AT Stockholm)
c.1274G>A, p.Arg425His (AT Glasgow)
c.1301T>C, p.Phe434Ser (AT Torino)
c.1318C>A, p.Pro439Thr (AT Budapest 5)
c.1349A>T, p.Asn450Ile
c.1367-1368del, c.1367-1371ins, p.Gly456delinsAlaThr
c.1384C>A, p.Pro461Thr

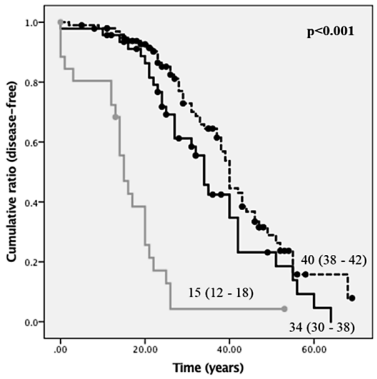
Fig. 2

A,



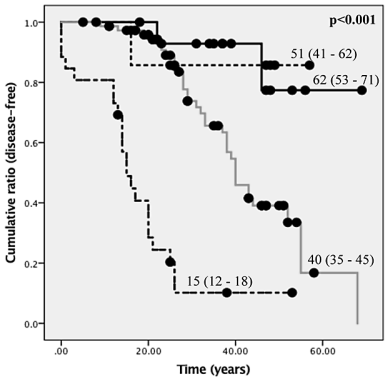
type I vs. type IIHBS HeZ	p=0.002
type I vs. type IIHBS HoZ	p<0.001
type IIHBS HeZ vs. type IIHBS HoZ	p<0.001

B,



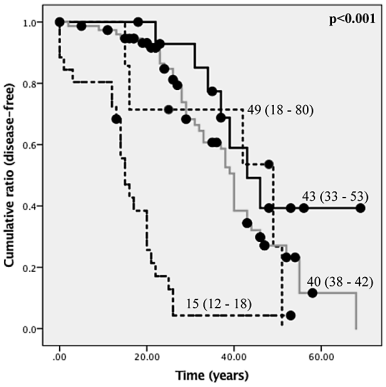
type I vs. type IIHBS HeZ	p=0.064
type I vs. type IIHBS HoZ	p<0.001
type IIHBS HeZ vs. type IIHBS HoZ	p<0.001

C,



AT Basel vs. AT Padua I	p=0.982
AT Basel vs. type ATBp3 HoZ	p=0.002
AT Basel vs. type ATBp3 HeZ	p =0.095
AT Padua I vs. ATBp3 HoZ	p<0.001
AT Padua I vs. ATBp3 HeZ	p=0.010
ATBp3 HoZ vs. ATBp3 HeZ	p<0.001

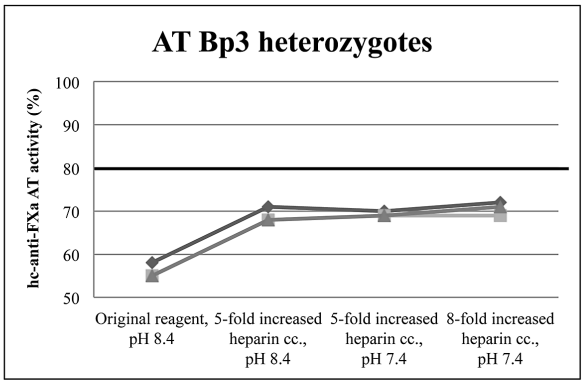
D,



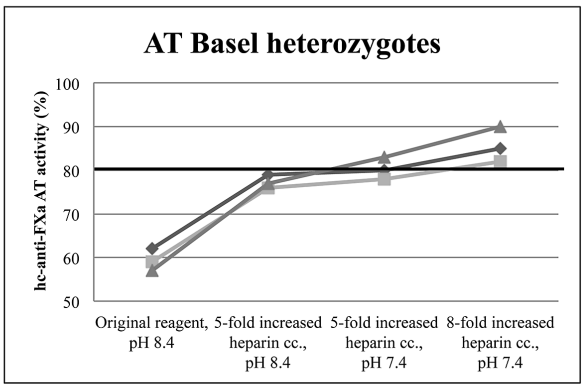
AT Basel vs. AT Padua I	p=0.459
AT Basel vs. ATBp3 HoZ	p=0.018
AT Basel vs. ATBp3 HeZ	p =0.997
AT Padua I vs. ATBp3 HoZ	p<0.001
AT Padua I vs. ATBp3 HeZ	p=0.130
ATBp3 HoZ vs. ATBp3 HeZ	p<0.001

Fig. 3

A,


AT Bp3 heterozygotes
hc-anti-FXa AT activity (%)
100
90
80
70
60
50
Original reagent, pH 8.4
5-fold increased heparin cc., pH 8.4
5-fold increased heparin cc., pH 7.4
8-fold increased heparin cc., pH 7.4

B,


AT Basel heterozygotes
hc-anti-FXa AT activity (%)
100
90
80
70
60
50
Original reagent, pH 8.4
5-fold increased heparin cc., pH 8.4
5-fold increased heparin cc., pH 7.4
8-fold increased heparin cc., pH 7.4

C,

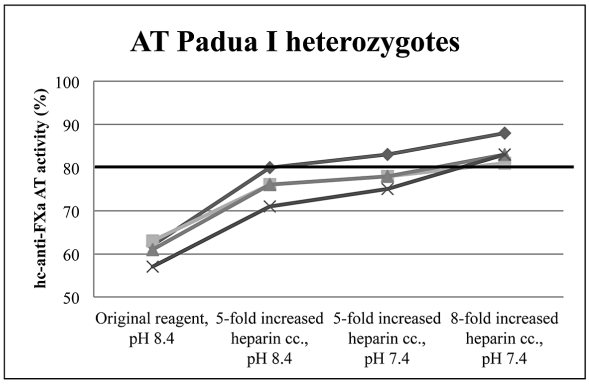
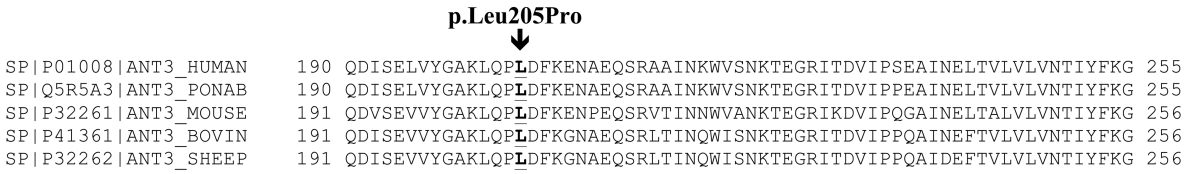

AT Padua I heterozygotes
hc-anti-FXa AT activity (%)
100
90
80
70
60
50
Original reagent, pH 8.4
5-fold increased heparin cc., pH 8.4
5-fold increased heparin cc., pH 7.4
8-fold increased heparin cc., pH 7.4

Fig. 4

A,


p.Leu205Pro
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SP|Q5R5A3|ANT3_PONAB 190 QDISELVYGAKLQPLDFKENAEQSRAAINKWVSNKTEGRITDVIPPEAINELTVLVLVNTIYFKG 255
SP|P32261|ANT3_MOUSE 191 QDVSEVVYGAKLQPLDFKENPEQSRVTINNWVANKTEGRIKDVIPQGAINELTALVLVNTIYFKG 256
SP|P41361|ANT3_BOVIN 191 QDISEVVYGAKLQPLDFKGNAEQSRLTINQWISNKTEGRITDVIPPQAINEFTVLVLVNTIYFKG 256
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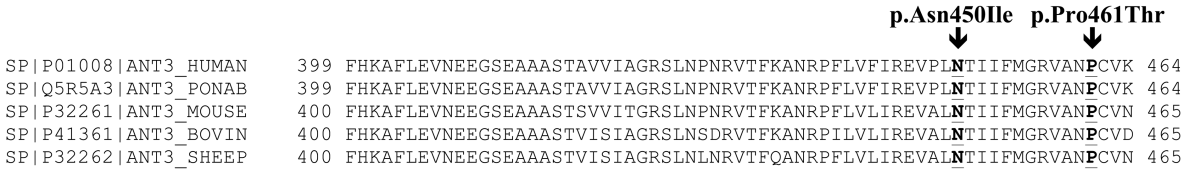
B,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p.Pro461Thr
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SP|Q5R5A3|ANT3_PONAB 399 FHKAFLEVNEEGSEAAASTAVVIAGRSLNPNRVTFKANRPFLVFIREVPLNTIIFMGRVANPCVK 464
SP|P32261|ANT3_MOUSE 400 FHKAFLEVNEEGSEAAASTSVVITGRSLNPNRVTFKANRPFLVLIREVALNTIIFMGRVANPCVN 465
SP|P41361|ANT3_BOVIN 400 FHKAFLEVNEEGSEAAASTVISIAGRSLNSDRVTFKANRPILVLIREVALNTIIFMGRVANPCVD 465
SP|P32262|ANT3_SHEEP 400 FHKAFLEVNEEGSEAAASTVISIAGRSLNLNRVTFQANRPFLVLIREVALNTIIFMGRVANPCVN 465