Antithrombin p.Thr147Ala, the first founder mutation from African origin responsible for inherited antithrombin deficiency

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Abstract

Background Hereditary antithrombin deficiency is a rare autosomal dominant disorder predisposing to recurrent venous thromboembolism (VTE). To date, only two founder mutations have been described.

Objectives We investigated the antithrombin p.Thr147Ala variant, found in twelve patients from African origin. This variant is known as rs2227606 with minor allele frequency of ~~1.00~~0.5% in Africans and absent in Europeans. A possible founder effect was investigated.

Methods Phenotypical characterization was established through immunological and functional methods, both in basal and stress-conditions. Recombinant antithrombin molecules were constructed by site-directed mutagenesis and expressed in HEK-293T cells. Secreted antithrombin was purified and functionally characterized. Structural modelling was performed to predict the impact of the mutation on protein structure. A novel nanopore sequencing approach was used for haplotype investigation.

Results Ten patients experienced VTE, stroke or obstetric complications. Antithrombin antigen levels and anti-IIa activity were normal or slightly reduced while anti-Xa activity was reduced with only one commercial assay. On crossed-immunoelectrophoresis, an increase of antithrombin fractions with reduced heparin affinity was observed under high ionic strength conditions but not in physiological conditions. The recombinant p.Thr147Ala protein displayed a reduced anti-Xa activity. Structural modelling revealed that residue Thr147 forms three hydrogen bonds that are abolished when mutated to alanine. The investigated patients shared a common haplotype involving 13 *SERPINC1* intragenic SNP's.

Conclusions Antithrombin p.Thr147Ala, responsible for antithrombin type II HBS deficiency, is the first founder mutation reported in people from African ancestry. This study further emphasizes the limitations of commercial methods to diagnose this specific subtype.

Introduction

Antithrombin is one of the most important physiological inhibitors of blood coagulation. This serine protease inhibitor (SERPIN) predominantly inhibits coagulation factor Xa and thrombin (factor IIa), a function that is significantly enhanced by heparin or by heparan sulfates present on the vessel wall.¹ The antithrombin gene, *SERPINC1*, encodes for a mature protein of 432 amino acids containing two important functional domains: the reactive site (RS), for binding with the target proteases and the heparin binding site (HBS), responsible for the interaction with heparin and heparan sulfates.²

Congenital deficiency of antithrombin is a rare autosomal dominant disorder with an estimated prevalence ranging from 0.02% to 0.2% in the general population. Deficiency of this natural anticoagulant is associated with a 16-fold increased risk for the development of venous thromboembolism (VTE) compared to individuals with normal antithrombin activity.³ Phenotypically, inherited antithrombin deficiency can be classified into quantitative (type I, reduced amount of antithrombin in plasma) or qualitative defects (type II, normal or slightly reduced amount of dysfunctional protein in plasma). Depending on the altered functional properties, type II deficiencies are subdivided into Reactive Site, Heparin Binding Site or Pleiotropic deficiencies (when the conformation of the molecule is affected).² In type I deficiencies, causative mutations are family-specific, while mutations responsible for type II deficiencies are often recurring due to a number of founder mutations.^{4, 5}

In the present study, we investigate the antithrombin variant, p.Thr147Ala, found in twelve patients of Black-African origin. This variant has been reported as a single nucleotide polymorphism (SNP) rs2227606 in the *SERPINC1* gene. *In silico* prediction tools for pathogenicity render conflicting results, therefore we initiated further studies to elucidate

the functional effect of this mutation. We hypothesize that this variant might be a pathogenic mutation, restricted to individuals from Black-African ancestry.

Materials and methods

Patient and controls

From January 1990 to April 2020, our center in Brussels recruited 578 blood samples from patients with suspicion of antithrombin deficiency, based on a reduced functional antithrombin activity measured in the referring center. Antithrombin activity analysis was repeated in our laboratory on an independent plasma sample and molecular analysis was performed to confirm the genetic defect. After exclusion of cases with acquired antithrombin deficiency and non-affected family members, a total of 421 inherited antithrombin deficient individuals remained in the study population.

In the present study, we focused on twelve patients carrying the rs2227606 polymorphism. We collected information concerning venous and arterial thrombosis, recurrent events, age at first and recurrent events, obstetrical history, ethnicity, presence of acquired risk factors for VTE (immobilization, surgery, oral contraceptive use, pregnancy, post-partum period, obesity), acquired risk factors for arterial thrombosis (obesity, smoking, hypertension), results of thrombophilia screening (lupus anticoagulant, anticardiolipin antibodies, anti- β_2 glycoprotein I antibodies, protein C activity, free protein S antigen, resistance to activated protein C, Factor V Leiden mutation and prothrombin G20210A mutation) and familial history of venous or arterial thrombosis.

A control population consisting of 100 subjects from African ancestry was randomly collected.

This study was approved by the Ethical Committee of the 'Universitair Ziekenhuis Brussel'

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Patient samples

Citrated plasma samples were obtained in absence of an acute thrombotic event or any antithrombotic treatment. Genomic DNA was extracted from peripheral blood leukocytes using standard methods.

Plasma assays

Antithrombin antigen levels were measured using Laurell rocket immunoelectrophoresis with a rabbit anti-human antithrombin antibody (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Antithrombin ~~anti-Xa~~functional activity was measured using three different commercial assays, all performed on an ACL TOP500 instrument (Werfen, Bedford, USA). Anti-Xa activity was determined with Innovance Antithrombin (Siemens Healthcare Diagnostics Products GmbH) and HemosIL Liquid AT (Werfen), according to manufacturer's instruction. The Innovance assay contains human FXa while the HemosIL Liquid AT contains bovine FXa. Using a third assay, Biophen AT anti-IIa (Hyphen Biomed, Neuville-sur-Oise, France), FIIa inhibition was measured with a short incubation time, as described previously.⁶ This assay uses bovine FIIa.

Antithrombin activity with varying incubation times was performed using a fourth assay, HemosIL Antithrombin (Werfen), also anti-Xa based. Briefly, reagents were reconstituted according to manufacturer's instructions and 50µL of plasma was incubated with 50µL of bovine factor Xa in the presence of heparin for 15, 3, 1 and 0 minutes. After addition of 50µL of chromogenic substrate, the absorbance was measured at 405nm with a Multiskan FC instrument (Thermo Fisher Scientific, Waltham, USA). Reactions were performed in

triplicate. We calculated the heparin binding ratio as described by Moore et al using the data of the 15 and 1 minute incubation time.⁷

Heparin affinity of plasma antithrombin variants was evaluated by crossed immunoelectrophoresis (CIE) in presence of heparin, as described elsewhere⁸⁷, but we used two different ionic strength conditions: 150 mM (physiological) and 0.8 M NaCl. Polyacrylamide gel electrophoresis (PAGE) was performed essentially as described elsewhere.⁹⁸

Molecular analysis

In patients, sequencing of all 7 exons and intron/exon boundaries of the *SERPINC1* gene was performed as previously described.⁶ Multiplex Ligation-Dependent Probe Amplification was performed in all patients carrying the rs2227606 polymorphism in order to exclude the presence of large deletions or insertions.

In control patients, only the presence of the rs2227606 polymorphism was investigated.

Mutations are described according to the Human Genome Variation Society Guidelines. The GenBank NM_000488 cDNA sequence was used as reference sequence.

We used ~~four~~ thirteen prediction tools to assess pathogenicity. These are listed in the supplementary material (Supplementary table 2). ~~:- PolyPhen-2, SIFT, ALIGN-GVGD and MutationTaster.~~

Haplotype analysis

To investigate a possible founder effect for the p.Thr147Ala variant, a novel approach based on nanopore sequencing was used. This technology does not require relatives to determine

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the haplotype, as it can find all genetic variants in the same chain of long reads generated by long-range PCRs.¹⁰ Detailed methodology can be found in the supplementary material.

Recombinant expression

Wild Type (WT) and mutant antithrombin p.Thr147Ala were constructed on the β -glycoform (p.Ser169Ala) background of antithrombin in order to reduce glycosylation heterogeneity. Site-directed mutagenesis was performed using the QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, USA) and the appropriate primers to obtain the p.Thr147Ala mutant on the pCEP4/AT-S169A plasmid, kindly provided by Dr. J. Huntington (Cambridge Institute for Medical Research, Cambridge, UK). Both WT and mutant plasmids were transfected into HEK-293T cells using jetPrime™ (Polyplus Transfection, New York, USA) as previously described.¹¹ Stable cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal-bovine serum (Invitrogen, Carlsbad, USA). Cells were grown to 70-80% confluence in cell factory systems before replacing the growth medium by Freestyle serum-free medium (Invitrogen). Cells were further grown at 37°C for three days followed by harvesting of the culture medium. The conditioned media were concentrated and dialyzed against the appropriate binding buffer (50mM Tris) with a 4MA ultrafiltration hollow-fiber cartridge (GE Healthcare, Waukesha, USA). Next, the dialyzed medium was loaded onto a HiTrap Heparin Column (GE Healthcare) coupled to an ÄKTA purifier (GE Healthcare). Fractions were eluted in a 50mM Tris-HCl (pH7.4) buffer by applying a NaCl gradient from 0M to 3M. Purity of the eluted fractions was assessed by reducing SDS-PAGE and Coomassie blue staining. Finally, positive fractions were pooled, dialyzed against PBS buffer and stored at -70°C.

We measured anti-Xa activity of both purified recombinant WT and mutant p.Thr147Ala with the Innovance Antithrombin assay (Siemens) and HemosIL Antithrombin (Werfen). The activity of the variant protein was normalized to the activity of the WT protein and expressed as percentages.

Functional studies

We evaluated formation of thrombin-antithrombin complexes, thermal stability, intrinsic fluorescence and performed structural modelling as described in the supplementary material.

Fluorescence studies

Equilibrium dissociation constants (K_d) for the antithrombin-heparin interaction of both recombinant WT and mutant p.Thr147Ala were determined essentially as described previously.¹²

Formation of thrombin-antithrombin (TAT) complexes

Plasma from the carriers of the p.Thr147Ala variant and controls was incubated with 0.2U of thrombin (Calbiochem, Merck KGaA) for 15 minutes at room temperature in presence of 5U of unfractionated heparin. These reactions result in the formation of TAT complexes that were detected by SDS-PAGE under reducing conditions followed by immunoblotting. These experiments were also performed on the recombinant proteins.

Thermal stability

Plasma of patients and controls was incubated at 40°C for 24 hours. Anti-Xa activity was measured before and after incubation using the HemosIL Antithrombin kit (Werfen) and the Innovance Antithrombin kit (Siemens) as described above.

Structural modelling

Structural modelling was performed using UCSF Chimera software (Resource for Biocomputing, Visualization, and Informatics, San Francisco, USA) using the antithrombin-FXa-pentasaccharide complex (PDB accession number 2gd4) as a template.

Statistics

Data are presented as mean $\pm$ standard deviation unless otherwise specified. Between-group comparisons were conducted using repeat measure one-way ANOVA. A p-value of 0.05 or less was considered to indicate statistical significance. Statistical and graphical analysis was performed with GraphPad Prism version 7.03 (GraphPad Software, San Diego CA, USA) and Analyse-it software version 2.26 (Analyse-it Software Ltd, Leeds, UK).

Results

Patients and controls

From the 421 genetically confirmed antithrombin deficient patients in our center in Brussels, twelve showed a heterozygous substitution of adenine to guanine at cDNA position 439 (c.439A>G) in exon 3 of the *SERPINC1* gene. This substitution leads to the replacement of threonine by alanine at amino acid position 147 (p.Thr147Ala). None of the carriers showed any other pathogenic mutations, large deletions or insertions.

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3 Interestingly, all twelve patients were unrelated and of Black-African origin: five originate
4 from Congo, two from Ruanda, two from Cameroun, one from Ivory Coast, one from Angola
5 and in a last patient the country of origin was not specified. Table 1 shows the clinical
6 presentation of the subjects.
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12 Eleven of the twelve subjects were female and 7 of them suffered from obstetric
13 complications (miscarriage, preeclampsia and failure to conceive). Five patients developed
14 venous thromboembolism and one experienced an arterial thrombotic event. Only one
15 patient reported a familial history of thrombosis. Thrombophilia screening was normal
16 except in patient 10, where reduced free protein S levels (54-57%) were measured at
17 different occasions. Genetic analysis of the *PROS1* gene in P10 showed a homozygous
18 p.Val510Met mutation, causative for protein S deficiency.
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22 The *SERPINC1* p.Thr147Ala variant is known as single nucleotide polymorphism (SNP)
23 rs2227606. Minor allele frequency (MAF) data extracted from Exome Aggregation
24 Consortium (ExAC), 1000 Genomes and gnomAD, showed a significant difference between
25 distinct ethnic groups. The SNP is nearly absent in Caucasian and Asian populations, whereas
26 its minor allele frequency in African population ~~reaches 1.0%~~ varies between 0.5 and 1.0%
27 (Supplementary table 1). We evaluated the prevalence of the variant in 100 randomly
28 selected subjects from African ancestry and identified 1 subject ~~with-carrying~~ the rs227606
29 variant in heterozygous state, confirming the ~~prevalence of 1%~~ allele frequency of 0.5%
30 (carrier frequency of 1%) in the African population. This subject also showed a reduced
31 antithrombin activity of 59%.
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35 *In silico* prediction tools designed to evaluate pathogenicity of sequence variations render
36 conflicting results (Supplementary table 2): six of the evaluated prediction tools classify this
37 variant as pathogenic (CADD, FATHMM, Mutation Taster, PolyPhen-2, PredictSNP2 and
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Provean), while the remaining seven consider this variant to be benign. PolyPhen-2 and MutationTaster classify the variant as 'possibly damaging' and 'disease-causing', respectively, while SIFT and ALIGN-GVGD predict that the variant is unlikely pathogenic.

According to the recommendations of the American College of Medical Genetics and Genomics (ACMG), this mutation should be classified as a 'variant of uncertain significance' (criteria for PM1, PM2 and PP2 rules are met).¹³ The PP3/BP4 rules addressing results from *in silico* tools cannot be used since this rule requires agreement between the different computational methods.

Plasma antithrombin assays

Antithrombin antigen levels were normal or slightly reduced (91.2%; range 70-110%) while anti-Xa activity was reduced with only one specific commercial test, Innovance Antithrombin (Figure 1). Activities measured with both HemosIL Liquid AT (anti-Xa based, 98.9 ± 10.6%) and Biophen AT anti-IIa (100.9 ± 10.2%) were significantly higher when compared to Innovance Antithrombin (61.8 ± 9.3%) and showed results within the normal range (80-120%). Based on activity-to-antigen ratio, these subjects show a qualitative defect or type II phenotype.

We and others have previously shown that incubation time of the plasma with FXa or thrombin plays a crucial role in the sensitivity of the activity assays for some specific type II mutations^{6, 146, 14}. To assess if this is the case for this variant, antithrombin anti-Xa activity was measured with HemosIL Antithrombin using different incubation times ranging from 0 to 15 minutes. Figure 2 shows an incubation time-dependent reduction in antithrombin activity in the plasma of the patients. Every shortening in incubation time (except t1 vs t0)

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3 resulted in a significant reduction of antithrombin activity. This effect was more pronounced
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5 when comparing the results of t0 to t15 ($p= 0.0002$) where we observed an average
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7 reduction of $22.6 \pm 9.2\%$. The antithrombin activity of normal human plasma remained
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9 constant when reducing incubation time. When using the incubation time proposed by the
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11 manufacturer, i.e. 3 minutes, only three out of the nine investigated patients would show an
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13 antithrombin activity below the reference range. The heparin-binding ratio of the patient
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15 plasma was clearly reduced when compared to the normal plasma, 0.78 and 1.03,
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17 respectively. This is indicative for a heparin binding site defect.
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25 We performed crossed-immunoelectrophoresis on the plasma of patient 5 (Figure 3). At
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27 physiological ionic strength, the pattern is similar to that of normal plasma. When treating
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29 the plasma with higher salt concentration in order to impair the binding of heparin to
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31 antithrombin, an antithrombin fraction with lower heparin affinity appears. This fraction did
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33 however not reach the levels that were observed in a heterozygous carrier of a Type II HBS
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35 deficiency such as Antithrombin Toyama (p.Arg79Cys).
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42 *Thermal stability*

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44 Plasma of four patients and four healthy controls was subjected to heat stress by incubation
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46 at 40°C for 24 hours. This induced an important reduction of anti-Xa activity in patients (29.8
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48 $\pm 12.0\%$) compared to the controls ($13.0 \pm 6.5\%$) when measured with HemosIL
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50 Antithrombin. The basal antithrombin activity was already reduced in patients when
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52 determined with Innovance Antithrombin and no further reduction was observed after heat
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54 stress (Figure 4).
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Heating of the plasma did not induce an increase of latent antithrombin or polymers (Supplementary Figure 2).

Recombinant expression

The p.Thr147Ala variant and WT antithrombins were produced in a recombinant model. The anti-Xa activity was measured using two different anti-Xa assays. The purified recombinant p.Thr147Ala protein displayed an anti-Xa activity of approximately 50% of the wild type recombinant antithrombin as shown in figure 5 ($51.9 \pm 2.2\%$ for Innovance Antithrombin Antithrombin and $55.0 \pm 3.3\%$ for HemosIL Antithrombin). Interestingly, anti-Xa activity was reduced with both assays, in contrast to the data observed in plasma samples. Heparin affinity was assessed by determining the equilibrium dissociation constants of the recombinant proteins for the pentasaccharide by following changes in intrinsic fluorescence. The dissociation constants (K_d values) for the wild type and mutant proteins were 1.18 ± 0.07 and 0.84 ± 0.06 nM, respectively, indicating no significant difference in heparin affinity.

TAT-complex formation

The formation of TAT-complexes, evaluated by SDS-PAGE under reducing conditions, showed identical results in carriers and controls (Supplementary figure 3). Similar results were observed with the recombinant proteins (data not shown).

Haplotype determination

Nine DNA samples of patients with the p.Thr147Ala mutation were available for haplotype analysis. LR-PCR amplicons were obtained in 8 out of the 9 carriers. These amplicons were sequenced for 20 minutes in the nanopore flow cell, and a mean of 367 and 460 reads, from

LR1-LR2 and LR3-LR6, respectively, were obtained. IGV and Illumina Variant Studio analyses revealed a profile of genetic variations identified in each allele, which included the same markers for the mutated allele.

This analysis revealed a common haplotype shared by all carriers of the mutation (Table 2).

Consistent with this result, read selection of reads carrying the p.Thr147Ala mutation Reads carrying the p.Thr147Ala mutation were selected by bioinformatics tools using 'grep' command in Linux and confirmed that ~~positive reads for~~ all patients shared the haplotype described in table 2. This common haplotype contains 13 small nucleotide variants whose localization and MAF in Caucasians and Africans is shown in Table 2.

Structural modelling

The affected residue Thr147 is a moderately conserved amino acid in the serpin superfamily, located at the bottom of helix D of the protein, in the loop connecting with helix C. This polar residue forms three hydrogen (H) bonds with amino acids Arg79, Ser144 and Glu145 (Figure 6A). Mutation of this amino acid into a non-polar alanine abolishes these three hydrogen bonds (Figure 6B).

The H-bond with Arg79 is of specific interest as this residue is known to directly interact with heparin. The interaction with Glu145 could also be of importance as this is a conserved residue among serpins.

Discussion

In this study, we have evaluated the functional consequences of the p.Thr147Ala antithrombin variant, identified in twelve unrelated patients with antithrombin deficiency

from Black-African origin. The majority of the patients were female (11/12) and suffered from obstetric complications (7/12). Thrombotic events were observed in six of the twelve patients, in half of them in the absence of acquired or transient thrombophilic risk factors.

The predominance of the p.Thr147Ala variant in female patients is remarkable as it is not observed in the data from gnomAD, ExAC and 1000 Genomes. The gender-bias observed in our study can be explained by the fact that females, unlike males, are exposed to specific thrombotic risk factors as pregnancy, hormone replacement therapy and pregnancy. Further studies are required to clarify whether the p.Thr147Ala variant is particularly associated with obstetric complications. Familial history of VTE was reported by only one patient, suggesting that this variant is associated with a mild thrombotic phenotype.

In all subjects, no other molecular defects were identified in the *SERPINC1* gene.

~~Two-Seven~~ of the ~~fourthirteen~~ *in silico* prediction tools for pathogenicity, ~~SIFT and ALIGN~~ ~~GVGD~~, classified this variant, also known as SNP rs2227606, as a benign polymorphism. A recent study evaluating different *in silico* methods in predicting pathogenicity of *SERPINC1* variants observed a large variability between several bioinformatics tools. Especially mutations causing Type II HBS deficiency were often misclassified as benign by the majority of the *in silico* tools.¹⁵¹⁵ The most powerful prediction tool in their study appeared to be PolyPhen-2, ~~whowhich~~ classified the p.~~Thr147Ala~~-Thr147Ala variant as 'possibly damaging'. However, in the same study, the p.Thr147Ala variant was regarded as a 'true negative', meaning that data on specificity and negative predictive values should be interpreted with caution. Another study found that predictions from five commonly used algorithms (PolyPhen-2, SIFT, CADD, Provean and MutationTaster) resulted in 79% concordance for pathogenic variants and only 33% for benign variants.¹⁶ Of these five algorithms, only SIFT predicted our variant to be tolerated.

Antithrombin activity in patient plasma was reduced only when measured with one specific commercial assay, Innovance Antithrombin. Combined with (nearly) normal antigen levels, these results suggest a Type II antithrombin deficiency. Our study provides further evidence of the limitations of commercially available methods to diagnose antithrombin deficiency. Indeed, it has already been reported that the sensitivity of certain commercial activity assays, especially thrombin-based ones, tends to be lower towards Type II HBS deficiencies.^{5, 6, 17-19, 6, 16-18} Reduction of incubation time of the plasma with thrombin or FXa can improve the sensitivity.^{6, 14, 20, 6, 14, 19} This was also the case in the present study where we observed a significant reduction of antithrombin activity (measured by the anti-Xa assay HemosIL Antithrombin) in the plasma from patients but not in normal controls. Heating of the patient plasma did also induce a reduction of the antithrombin activity when measured with the same assay.

Additional evidence for the pathogenic consequences of the p.Thr147Ala was obtained from the studies done on recombinant proteins. The recombinant p.Thr147Ala showed approximately 50% anti-FXa inhibition of the WT antithrombin but without altered heparin affinity, as shown by fluorescence studies. The fact that we could not show any effect on heparin affinity in the recombinant protein could be explained by the use of the β -glycoform background. Two antithrombin glycoforms are found in plasma: first, the α -isoform that represents the main circulating fraction (90%) and contains four N-glycans; second the β -isoform, that lacks the N-glycan at position 135, is the minor fraction (10%). Of these two glycoforms, β -antithrombin has the higher affinity for heparin and thus functions as the major inhibitor *in vivo* even though it is the less abundant form.^{21, 20} A previous study has shown that the β -isoforms of HBS variants retain their heparin affinity and may also compensate for the effect of mutations causing heparin-binding defects.^{22, 21} In our study, the

recombinant proteins were constructed on the β -isoform, possibly explaining the preserved heparin affinity. Moreover, the anti-Xa activity of the recombinant p.Thr147Ala protein was reduced with both assays, contrasting with the data observed in plasma. When measured with the HemosIL Antithrombin assay, plasma of patients carrying the p.Thr147Ala variant shows normal antithrombin activity, while recombinant, pure β -p.Thr147Ala displays reduced antithrombin activity. This suggests that the effect of the p.Thr147Ala variant on the anticoagulant function of antithrombin is more pronounced on the β -glycoform.

Structural modelling revealed that residue Thr147 forms three hydrogen bonds that are all abolished when mutated to Ala. The H-bond with Arg79 is of specific interest as this residue is known to directly interact with heparin and mutations of this residue result in a loss of affinity towards heparin.^{23, 2422, 23} We speculate that the absence of this hydrogen bond causes a distortion of the heparin binding site and possibly leads to a misalignment of the pentasaccharide. This is further supported by the results of the CIE. Under physiological conditions, no difference is observed between normal and patient plasma. However, under higher ionic strength conditions, an increase in low-affinity variants is observed. Hydrogen bonds are known to be affected by higher ionic strength and the fact that the p.Thr147Ala mutation already induces a loss of three H-bonds, only exacerbates this effect. Another stress condition known to disturb hydrogen bonds is temperature. As already discussed, heating of the patient plasma reduced the (apparently normal) activity compared to controls when measured with the HemosIL Antithrombin kit. We speculate that the reaction conditions (pH, incubation time, heparin concentration) of the Innovance Antithrombin kit, are mimicking a stress condition making it the only commercial assay in this study able to detect the functional (and pathological) consequences of p.Thr147Ala variant. Moreover,

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3 this assay uses human FXa in contrast to the other anti-Xa assays in this study where bovine
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5 FXa is used.
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8 Threonine 147 is located on the bottom of helix D, a region highly involved in binding to the
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10 pentasaccharide of heparin. Upon binding of antithrombin to heparin, several structural
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12 rearrangements occur, like the rotation and extension of helix D.²⁴²³ Mutations in amino
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14 acids sterically surrounding residue 147 have been proven to highly disturb or even abolish
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16 heparin affinity.^{25, 2624, 25} Picard et al. reported a female patient with antithrombin deficiency
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18 carrying a different mutation on the same amino acid residue, p.Thr147Ile. In this patient,
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20 extremely low activity was measured (25%) in combination with an antigen of 50%. The
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22 possible hypothesis of homozygosity or compound heterozygosity could however not be
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24 proven. The patient and her twin sister suffered from spontaneous abortion, a clinical
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26 presentation that is shared by four of the patients reported in our study.²⁷²⁶
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28 Interestingly, the p.Thr147Ala variant was only identified in individuals from Black-African
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30 origin. By using an original approach with nanopore sequencing of long-range PCRs that does
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32 not require analysis of relatives, we demonstrate a common haplotype in all carriers. This
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34 haplotype contains 13 intragenic markers, some of them with very low MAF, strongly
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36 supporting a founder effect. So far only two founder mutations have been described in
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38 *SERPINC1*, both also responsible for type II HBS deficiencies: Antithrombin Budapest 3
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40 (p.Leu131Phe) in Hungary and antithrombin Basel (p.Pro73Leu) in the Finnish population.^{4, 5}
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42 We report the first *SERPINC1* founder mutation identified in the Black-African population.
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44 Further studies are required to determine at what evolutionary time point this variant, that
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46 has a relatively high prevalence in Africans, has occurred. According to the 1000Genomes
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48 data obtained from the African/African American population data set of 5203 individuals
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50 (3315 female and 1888 male samples) assembled by the Exome Aggregation Consortium, it is
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close to 1%, strongly suggesting that it originated a long time ago during evolution and has a wide distribution in this continent. Our own data confirm the prevalence of 1% in individuals from Black African ancestry. ~~It is conceivable that the p.Thr147Ala variant is also highly prevalent among African-Americans. Although the antithrombin p.Thr147Ala variant is predominantly found in Africans, it was also identified in African-Americans and Europeans, but in much lower numbers. This can be explained by the recent acceleration and geographical diversification (beyond colonial patterns) of migration from Africa to Europe, but increasingly also to North America.~~²⁸²⁷ Results from a recent meta-analysis show that individuals from African descent appear to have a higher risk for the development of VTE when compared to Caucasians (OR 1.28, 1.19-1.38 95% CI).²⁹²⁸ While the Factor V Leiden and prothrombin G20210A variant are absent in people from Black-African ancestry, the prevalence of deficiencies of the natural anticoagulants, protein C, protein S and antithrombin, is reported to be equal in both ethnic groups.³⁰⁻³²²⁹⁻³¹ It was hypothesized that the higher prevalence of VTE among people from African ancestry might be due to an increased prevalence of acquired risk factors for VTE but this could not be confirmed.³⁰²⁹ The authors of the same study suggest the underlying role of yet unknown genetic components. In the light of our present findings, we presume that the p.Thr147Ala variant in the *SERPINC1* gene could be such a candidate. Moreover, this variant is hardly detected by commercial assays evaluating antithrombin anticoagulant activity and thus probably underreported. Interestingly, the *PROS1* mutation, p.Val510Met, identified in patient 10, was shown to be a population-specific risk factor for VTE, restricted to individuals from African descent.³³³² Analogous to the hypothesis of a positive selection explaining the high prevalence of common prothrombotic polymorphisms like Factor V Leiden among Caucasians³⁴³³, we speculate that in Africans other mild prothrombotic mutations with possible health benefits

might be selected. These polymorphisms, including the p.Thr147Ala variant in *SERPINC1* for the African population, could predispose to different advantageous phenotypes. Beneficial effects of Factor V Leiden on hemostasis (less hemorrhagic events during delivery), on inflammation (higher survival rate in and lower susceptibility to severe sepsis) and even on fertility (higher fecundity in both men and women) could underlie the selection.³⁴³³ It is hypothesized that these beneficial effects might not be driven by the Factor V Leiden mutation itself but rather by (not-yet identified) polymorphisms in surrounding genes that are co-inherited with Factor V Leiden (a mechanism called genetic hitchhiking).³⁵³⁴ As F5 and SERPINC1 are located in close proximity on the long arm of chromosome 1, it is conceivable that the observations for the Factor V Leiden variant can be extrapolated to the antithrombin p.Thr147Ala variant and thus support its positive selection. However, in the modern and aging Western society with increased risk of thrombosis such as oral contraceptive use or sedentary habits, these originally 'positive' defects would show their pathogenic reverse side, revealing new evidences of the complex and sensitive equilibrium of the hemostatic system.

The present study has investigated the p.Thr147Ala variation in the *SERPINC1* gene. We could demonstrate that the variant is a pathogenic founder mutation responsible for an inherited type II HBS antithrombin deficiency. As the mutation is restricted to people from African descent, further studies including large case control studies in populations from African ancestry are required to determine the exact risk of thrombosis and bad pregnancy outcome associated with this variant.

Conflict of interest

The authors state that they have no conflict of interest to declare.

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For Peer Review

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Figure Captions

Figure 1: Antithrombin activity measured with 3 different commercial assays. Dashed line: lower limit of normal range

Figure 2: Antithrombin activity measured with HemosIL Antithrombin and different incubation times. Full lines: patient plasma, Dotted line: normal pooled plasma. * indicates statistical significant difference. Dashed line: lower limit of normal range

Figure 3: Crossed immunoelectrophoresis of the plasma of patient 5. Plasma of a patient with Type II HBS (p.Arg79Cys) and a normal plasma sample were used as controls. A) Physiological ionic strength (150mM NaCl). B) High ionic strength (0.8M NaCl).

Figure 4: Antithrombin activity in patients and controls in basal conditions and after incubation for 24h at 40°C. Dashed line: lower limit of normal range

Figure 5: Antithrombin activity of the recombinant antithrombin proteins using two different anti-Xa activity assays. Dashed line: lower limit of normal range

Figure 6: 3D modelling of antithrombin. A: Wild Type antithrombin with residue 147 (Thr) depicted in yellow. B: Mutant antithrombin with residue 147 (Ala) shown in green. Blue lines represent H-bonds. Heparin is shown in stick representation, antithrombin in ribbon.

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Tables

Table 1: Clinical characteristics of the patients included in the study

Patient	Sex	Clinical presentation			Familial history of VTE
		Age	Event	Risk factor(s)	
P1	F	29	Pulmonary embolism	OC, obesity	+
		31	Miscarriage at 12 weeks gestation (thromboprophylaxis: nadroparine 0.6mL/d)	Obesity	
P2	F	ns	Incidental finding of non-recent portal vein thrombosis	None	-
P3	F	ns	2 early miscarriages	/	-
		30	Severe preeclampsia (neurological complications, coma, epilepsy) at 5 months gestation of twin pregnancy, loss of 1 foetus	Hypertension	
P4	F	40	CVA (occlusion of left arteria cerebri media) with hemiplegia and aphasia	None	-
P5	F	ns	3 early miscarriages	Obesity	-
P6	F	ns	None (incidental finding in context of fertility work-up)	None	-
P7	F	60	Proximal DVT of right leg	Obesity	-
P8	F	38	None (incidental finding in context of fertility work-up)	None	?
P9	M	35	Thrombophlebitis of left leg	/	?
		60	Proximal DVT of left leg	Surgery (renal transplant) + corticosteroid use	
		62	Thrombosis of arteriovenous fistula	Tamoxifen treatment + corticosteroid use	
		64	Proximal DVT of left leg	Tamoxifen treatment +	

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				corticosteroid use	
P10	F	43	Pulmonary embolism	None <u>Protein S</u> <u>deficiency</u>	-
P11	F	26	Early miscarriage	None	-
		38	Transient ischemic attack	Recent long haul flight	
P12	F	24	Preterm delivery due to gestational hypertension	Obesity	-
		27	Stroke-like episode	Pregnancy, Obesity	

CVA: cerebral vascular accident, DVT: deep vein thrombosis, F: female, M: male, OC: oral contraception, ns: not specified

Table 2: Single Nucleotide Variations linked to the p.Thr147Ala variation (*italic bold*) in *SERPINC1* located on the same allele.

dbSNP ID	Coordinate	Change	Localization	Codon	Missense change	MAF* (%)	MAF* (%)
	(chrom 1)#					Caucasian	African
rs2227611	173875467	A>G	Intron variant	--	--	0.3	27.6
rs138511779	173877033	C>T	Intron variant	--	--	0.0	26.0
rs1799876	173878471	A>G	Intron variant	--	--	33.1	83.5
rs5878	173878832	T>C	Exon 5	337	Q (synonymous)	34.7	84.4
rs5877	173878862	T>C	Exon 5	327	V (synonymous)	34.0	83.7
rs2227610	173879178	T>C	Intron variant	--	--	0.0	2.0
rs2227606	173881122	T>C	Exon 3	147	T/A	0.0	1.0
rs941989	173881871	C>T	Intron variant	--	--	34.2	84.4
rs2227601	173882730	T>C	Intron variant	--	--	12.3	68.5
rs2227597	173884739	C>G	Intron variant	--	--	12.1	44.8
rs2227596	173884793	T>C	Intron variant	--	--	15.9	78.7
rs2227594	173885481	C>T	Intron variant	--	--	12.1	44.8
rs2227593	173885536	G>T	Intron variant	--	--	12.1	44.9
rs2227590	173886037	G>A	Intron variant	--	--	12.1	46.9

According to GRCh37/hg19.
* Minor allele frequency according to 1000 Genomes.

What is known about this topic?

- Inherited antithrombin (AT) deficiency is a well-known risk factor for venous thromboembolism
- Genetic risk factors for thrombosis vary between different ethnic groups
- Very few founder mutations responsible for inherited antithrombin deficiency have been reported.

What does this paper add?

- We report antithrombin p.Thr147Ala, the first founder mutation from African origin causing inherited antithrombin deficiency.
- Antithrombin p.Thr147Ala can be regarded as an additional risk factor for thrombosis, restricted to patients from African ancestry.
- The variant is probably underreported due to lack of sensitivity of commercial activity assays

Figure 1: Antithrombin activity measured with 3 different commercial assays. Dashed line: lower limit of normal range

131x115mm (600 x 600 DPI)

Figure 2: Antithrombin activity measured with HemosIL Antithrombin and different incubation times. Full lines: patient plasma, Dotted line: normal pooled plasma. * indicates statistical significant difference. Dashed line: lower limit of normal range

144x113mm (150 x 150 DPI)

Figure 3: Crossed immunoelectrophoresis of the plasma of patient 5. Plasma of a patient with Type II HBS (p.Arg79Cys) and a normal plasma sample were used as controls. A) Physiological ionic strength (150mM NaCl). B) High ionic strength (0.8M NaCl)

232x129mm (150 x 150 DPI)

Figure 4: Antithrombin activity in patients and controls in basal conditions and after incubation for 24h at 40°C. Dashed line: lower limit of normal range

190x67mm (600 x 600 DPI)

Figure 5: Antithrombin activity of the recombinant antithrombin proteins using two different anti-Xa activity assays. Dashed line: lower limit of normal range

183x88mm (300 x 300 DPI)

Figure 6: 3D modelling of antithrombin. A: Wild Type antithrombin with residue 147 (Thr) depicted in yellow. B: Mutant antithrombin with residue 147 (Ala) shown in green. Blue lines represent H-bonds. Heparin is shown in stick representation, antithrombin in ribbon