Thrombosis Research 175 (2019) 68–75

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

SERPINC1 variants causing hereditary antithrombin deficiency in a Danish population☆

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ARTICLE INFO

Keywords:
Antithrombin III deficiency
Antithrombin deficiency type 2
SERPINC1
Factor V Leiden
Venous thromboembolism

ABSTRACT

Introduction: Antithrombin deficiency is associated with increased risk of venous thromboembolism (VTE). We aimed to identify variants causing antithrombin deficiency in a Danish population.

Materials and methods: We performed Sanger sequencing and, in relevant cases, multiplex ligation-dependent probe amplification analyses, in 46 individuals (23 index cases) with and 9 relatives without antithrombin deficiency. Furthermore, in order to explore whether a combination of antithrombin type II heparin binding site (HBS) deficiency and factor V Leiden single nucleotide variant (SNV) conferred a higher risk of VTE than either risk factor alone, we performed genotyping for factor V Leiden in most of the carriers of type II HBS deficiency (n = 25).

Results: We detected causal variants in all 46 carriers: three large and two small deletions, all causing type I antithrombin deficiency, and seven SNVs: one causing type I, one causing type II reactive site (RS), four causing type II HBS and one causing pleiotropic effect (PE) type II antithrombin deficiency. None of the relatives without antithrombin deficiency had the family variant. All detected SNVs have been reported previously. Majority (n = 27) of carriers had type II HBS deficiency, most often caused by the p.(Pro73Leu) SNV (n = 19). Heterozygosity for factor V Leiden was observed in three (3/25 = 12%) carriers of type II HBS deficiency. Only four (4/25 = 16%) carriers of type II HBS antithrombin deficiency experienced VTE, and two of these were heterozygous for factor V Leiden.

Conclusions: In a systematic search to identify variants causing hereditary antithrombin deficiency in a Danish population, we achieved a variant detection rate of 100%.

1. Introduction

Antithrombin is a major circulating inhibitor of thrombin, and to a lesser extent coagulation factor Xa and other blood coagulation proteases [1,2]. It is produced in the liver as a 464 amino acid precursor, from which a 32-amino acid signal peptide is cleaved off before secretion [1,2]. It folds into three β-sheets (A, B and C) and nine α-helices (A-I) (Fig. 1). Antithrombin includes two functional domains: the C-terminal reactive site (RS) and the N-terminal heparin binding site (HBS) [1,2]. Proteases such as thrombin and coagulation factor Xa attempt to cleave antithrombin at the RS. This induces conformational changes in antithrombin, and results in entrapment of the protease [1]. Thus, one antithrombin molecule inhibits one protease molecule. Consequently, individuals with decreased antithrombin activity have an approximately 16-fold increased risk of venous thromboembolism compared with individuals with normal antithrombin activity [3]. The inhibitory activity of antithrombin is enhanced by the presence of heparin. Binding of heparin to the HBS increases the rate of protease inhibition by a 1000-fold [2]. This explains the therapeutic use of heparin for prophylaxis and treatment of venous thromboembolism.

Abbreviations: SERPINC1, serpin peptidase inhibitor, Clade C Member 1; HBS, heparin binding site; RS, reactive site; PE, pleiotropic; MLPA, multiplex ligation-dependent probe amplification analysis; DHPLC, denaturating high performance liquid chromatography; ExAC, exome aggregation consortium; PolyPhen-2, polymorphism phenotyping v2; PhD-SNP, predictor of human deleterious single nucleotide polymorphisms; MutPred2, mutation predictor version 2.; AA, amino acid; NA, not applicable; ND, not detected; VTE, venous thromboembolism; PE, pulmonary embolism

☆ Declarations of interest: none.

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https://doi.org/10.1016/j.thromres.2019.01.022
Received 25 September 2018; Received in revised form 8 January 2019; Accepted 30 January 2019
Available online 31 January 2019
0049-3848/

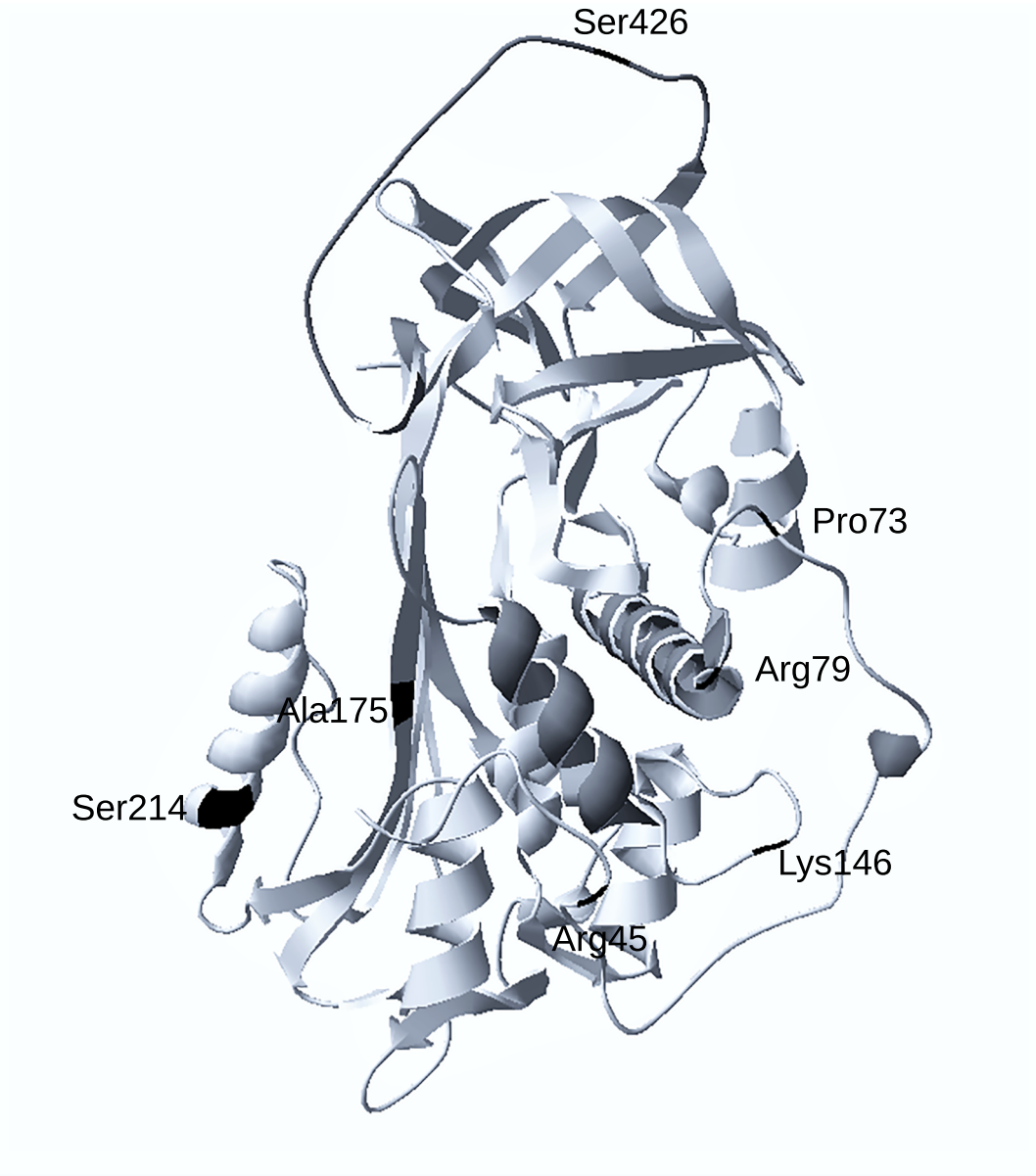

Fig. 1. Structural image of antithrombin with locations of identified single nucleotide variants.
The reactive site (RS) is illustrated at the top of the figure, where the residue Ser426 is located. The heparin binding (HBS) site is illustrated on the right side of the figure, where the residues Arg45, Pro73, Arg79 and Lys146 are located.

Heritable antithrombin deficiency is a rare autosomal dominant disorder, with a prevalence estimated to be 1% in patients with venous thromboembolism [2], and 0.2% in the general population [4–6]. The human antithrombin gene, *SERPINC1*, is located on the long arm of chromosome 1, at 1q25.1, and spans 13.5 kb, of which the major transcript NM_000488.3 contains seven exons [2]. More than 300 genetic variants causing antithrombin deficiency have been identified for this gene [2].

Antithrombin deficiency is classified into type I and type II [2]. In type I deficiency, both antigen and activity levels are reduced, indicating that the protein is not produced, or is being cleared faster due to the mutant allele. In type II deficiency, antigen levels are normal, while activity is reduced, indicating that a functionally defective protein is produced by the mutant allele. Type II can be further subdivided into three types based on the location of variants in functional domains: type II RS, type II HBS and type II PE (pleiotropic). This classification has clinical importance because different types are associated with different risks of venous thromboembolism [7].

Type I is associated with greater risk of venous thromboembolism than type II [7]. This is further supported by the fact that the most common deficiency type in the general population is type II HBS, while in populations of patients with venous thromboembolism, type I is more common [2].

However, there is a substantial variability in risk of venous thromboembolism for individuals with antithrombin type II HBS deficiency [8]. It has been suggested that this variability possibly could be explained by a coexistence of another relatively common genetic risk factor, such as factor V Leiden [9]. Factor V Leiden is a single nucleotide variant in the coagulation factor V gene, which eliminates one of its three activated protein C cleavage sites, and results in an increased risk of venous thromboembolism [10]. This single nucleotide variant is present in 8% of the Danish general population [11]. Because *SERPINC1* and the coagulation factor V gene are located close to each other, (at 1q25.1 and 1q24.2, respectively) they may co-segregate, leading to a higher risk of venous thromboembolism than either risk factor alone. However, due to very few individuals with a combination of

antithrombin deficiency and factor V Leiden, present evidence is not clear on whether the combination of these genetic risk factors confers a higher risk of venous thromboembolism than either risk factor alone [9,12–14].

The aim of this study was to identify variants causing antithrombin deficiency in a Danish population. In search of these variants, we performed Sanger sequencing and, in relevant cases, multiplex ligation-dependent probe amplification analyses (MLPA) in individuals previously diagnosed with antithrombin deficiency and their relatives without antithrombin deficiency. Furthermore, in a post hoc analysis, in order to explore whether a combination of antithrombin type II HBS deficiency and factor V Leiden single nucleotide variant conferred a higher risk of venous thromboembolism than either risk factor alone, we performed genotyping for factor V Leiden in most of the carriers of type II HBS deficiency.

2. Materials and methods

2.1. Participants

The study was performed at the Centre for Hemophilia and Thrombosis, Department of Clinical Biochemistry, Aarhus University Hospital, Denmark. All individuals referred to the department due to a cardiovascular event (venous thromboembolism, arterial thrombosis), obstetrical complications, family history (of cardiovascular events or antithrombin deficiency) or other reasons (incidental finding, etc.), and diagnosed with antithrombin deficiency were invited to participate in this study. The individuals were identified through the department's journal records and contacted by letter or during ambulatory visit. The ethical approval of the study did not allow identification and direct contact to the first-degree relatives of individuals with antithrombin deficiency. Therefore, we informed all the participants that all their first-degree relatives were welcome to participate regardless whether they had antithrombin deficiency or not. Because antithrombin deficiency is an autosomal dominant defect with full penetrance, we expected the relatives with normal antithrombin activity not to carry the family variant. In other words, we used relatives without antithrombin deficiency as controls. In a post hoc analysis, we asked all carriers of type II HBS deficiency for whom we did not have information on factor V Leiden genotype, for permission to perform this analysis as well. Majority (25 out of 27) responded and gave their consent.

We collected information based on medical records on diagnoses of venous thromboembolism, and did not collect information on diagnosis of arterial thrombosis.

2.2. Ethics

All participants gave written informed consent. The study was approved by The Central Denmark Region Committees on Health Research Ethics (No. 1-10-72-333-14), and performed according to the declaration of Helsinki.

2.3. Antithrombin measurements

Antithrombin activity was measured by a chromogenic assay using human FXa and bovine serum albumin on automated standard laboratory equipment (Sysmex CS 5100i, Siemens healthineers, Erlangen, Germany). The reference interval for antithrombin activity was 80–120%.

2.4. DNA analyses

DNA was isolated from blood EDTA stabilized samples by use of either the QiaSymphony DSP mini kit (Qiagen, Hilden, Germany) or the Maxwell 16 Blood DNA Purification Kit (Promega, Nacka, Sweden) according to the manufacturer's instructions. EDTA blood and DNA samples were stored at −20 °C until analysis. Seven PCR fragments were amplified, covering all 7 exons and adjacent splice sites, according to a previously described method [15]. The annealing temperature was 59 °C for exon 6, while it was 55 °C for all other exons. We used laboratory's standard PCR conditions and Ampli*Taq*-Gold DNA polymerase (Life Technologies Europe BV). After ethanol precipitation and wash, the sequences were separated on an Applied Biosystems 3500 or 3500xl Genetic analyzer (Life Technologies Europe BV). Sequence traces were aligned to reference sequence NM_000488.3 using SeqScape software (version 2.7, Life Technologies Europe BV). Variants were named based on the NM_000488.3 reference sequence according to guidelines of the Human Genome Variation Society [16]. Every variant was confirmed independently by a second run. Relatives without antithrombin deficiency were only tested for the family variant.

Individuals, in whom no variant was detected by sequencing, were subjected to further DNA analyses by multiplex ligation-dependent probe amplification analysis (MLPA). This was performed using SALSA MLPA probemix P227-B2 *SERPINC1* kit (MRC-Holland, Amsterdam, Netherlands) according to manufacturer's instructions. This kit detects deletions and duplications of one or more sequences in *SERPINC1*. DNA samples from normal individuals were used as controls in each run. Heterozygous deletions of recognition sequences are expected to give a 35–50% reduced relative peak height of the amplification product of the exon-specific probe. Every deletion was confirmed by a second run.

Genotyping for factor V Leiden (NM_000130.4:c.1610G > A, rs6025) was performed by high resolution melting (HRM). Briefly, asymmetrical PCR was performed with LightScanner Mastermix (BioFire Defense, Salt Lake City, USA), and HRM was performed with a LightScanner HR96 (BioFire Defense). Primer and probe sequences are available on request.

2.5. Data analysis

All detected single nucleotide variants were evaluated for the possible impact of the amino acid substitutions on the structure and function of antithrombin using three bioinformatic tools: PolyPhen-2 (URL: http://genetics.bwh.harvard.edu/pph2/), PhD-SNP (URL: http://snps.biofold.org/phd-snp/phd-snp.html) and MutPred2 (http://mutpred2.mutdb.org/index.html#qform).

In order to evaluate their prevalence, we performed a search for detected variants in the Exome Aggregation Consortium (ExAC). This database spans 60,706 unrelated individuals sequenced as part of various disease-specific and population genetic studies (URL: http://exac.broadinstitute.org/.)

We used PdbViewer (Swiss-PdbViewer at https://spdbv.vital-it.ch/) for producing structural image of antithrombin (protein identification: 1T1F) with locations of identified single nucleotide variants (Fig. 1).

Statistical analyses were performed using STATA 13.1. Fisher's exact test or Cuzick's nonparametric test for trend was used for comparisons.

3. Results

The study included 46 individuals with inherited antithrombin deficiency from 23 families and 9 relatives without antithrombin deficiency from 6 of the families.

We detected a causal variant in every single carrier of antithrombin deficiency, while none of the relatives without antithrombin deficiency had the family variant.

Twelve different variants were detected: three large deletions, two small deletions: p.(Asn159ProfsTer28) and p.(Glu277ValfsTer20), and seven single nucleotide variants: p.(Ser214Phe), p.(Ser426Leu), p.(Arg45Trp), p.(Pro73Leu), p.(Arg79His), p.(Lys146Glu) and p.(Ala175Asp) (Table 1). All seven single nucleotide variants have been reported previously: five of them had a dbSNP ID number, and three were observed in the ExAC database (p.(Arg45Trp), p.(Pro73Leu) and p.(Arg79His)). All single nucleotide variants were evaluated to be likely

Table 1
SERPINC1 variants in a Danish population with antithrombin deficiency.

Variant (exon location)	dbSNP ID number	Nucleotide change	AA change	ExAC, frequency	Participants (families), N	Relatives[a] (families), N	Type of deficiency	Antithrombin activity, %	Age[b], mean (range) in years	Age at first VTE, years	VTE, N (%)	Recurrent VTE, N (%)	¤PolyPhen-2 PhD-SNP MutPred2	Ref.
Large deletion (exon 1–4)	NA	NA	NA	ND	2 (1)	NA	I	53 (45–60)	50 (49–50)	21, 40	2 (100)	2 (100)	Not applicable	Novel
Large deletion (exon 1–5)	NA	NA	NA	ND	4 (1)	NA	I	57 (55–60)	47 (26–61)	17,19, 40	3 (75)	2 (50)	Not applicable	Novel
Large deletion (exon 5–6)	NA	NA	NA	ND	1 (1)	NA	I	52	41	16	1 (100)	0 (0)	Not applicable	Novel
Small deletion (exon 3)	NA	475_479delAACTG	Asn159ProfsTer28	ND	1 (1)	NA	I	50	62	24	1 (100)	0 (0)	Not applicable	Novel
Small deletion (exon 5)	NA	830_831delAG	Glu277ValfsTer20	ND	2 (1)	1 (1)	I	56 (48–64)	44 (26–62)	NA	0 (0)	0 (0)	Not applicable	Novel
SNV (exon 4)	NA	641C > T	Ser214Phe	ND	3 (1)	1 (1)	I	57 (51–65)	31 (19–51)	20, 28	2 (67)	0 (0)	Likely pathogenic Likely pathogenic Likely pathogenic	19
SNV (exon 7)	rs121909550	1277C > T	Ser426Leu	ND	5 (2)	NA	II RS	64 (58–70)	35 (19–48)	16, 18, 36	3 (60)	1 (20)	Likely pathogenic Likely pathogenic Likely pathogenic	20
SNV (exon 2)	rs768704768 (+rs121909551)	133C > T (+218C > T)	Arg45Trp (+Pro73Leu)	0.003% (NA)	2 (1)	NA	II HBS	23 (22–24)	42 (38–45)	NA	0 (0)	0 (0)	Likely pathogenic Likely pathogenic Likely pathogenic	23
SNV (exon 2)	rs121909551	218C > T	Pro73Leu	0.14%	17 (9)	5 (3)	II HBS	57(47–68)	45 (21–72)	25, 46, 60	3 (18)	0 (0)	Likely pathogenic Likely pathogenic Likely pathogenic	24–25
SNV (exon 2)	rs121909552	236G > A	Arg79His	0.01%	4 (2)	NA	II HBS	53 (48–59)	58 (38–67)	35	1 (25)	0 (0)	Likely pathogenic Likely pathogenic Likely pathogenic	21

(*continued on next page*)

Table 1 (*continued*)

Variant (exon location)	dbSNP ID number	Nucleotide change	AA change	ExAC, frequency	Participants (families), N	Relatives[a] (families), N	Type of deficiency	Antithrombin activity, %	Age[b], mean (range) in years	Age at first VTE, years	VTE, N (%)	Recurrent VTE, N (%)	¤PolyPhen-2 PhD-SNP MutPred2	Ref.
SNV (exon 3)	rs1170430756	436A > G	Lys146Glu	ND		NA	II HBS	53 (47–62)	44 (36–53)	NA	0 (0)	0 (0)	Likely pathogenic Likely pathogenic Benign	22–23
SNV (exon 3)	NA	524C > A	Ala175Asp	ND		NA	II PE[c]	61	37	NA	0 (0)	0 (0)	Likely pathogenic Likely pathogenic Likely pathogenic	17

The Exome Aggregation Consortium (ExAC) spans 60,706 unrelated individuals sequenced as part of various disease-specific and population genetic studies (URL: http://exac.broadinstitute.org/.) [August 2018].
SNV: single nucleotide variant. AA: amino acid. NA: not available. ND: not detected. Ref.: literature reference.
¤All detected single nucleotide variants were evaluated for the possible impact of the amino acid substitutions on the structure and function of antithrombin using three bioinformatic tools: PolyPhen-2 (URL: http://genetics.bwh.harvard.edu/pph2/), PhD-SNP (URL: http://snps.biofold.org/phd-snp/phd-snp.html) and MutPred2 (http://mutpred2.mutdb.org/index.html#qform). The results are listed in that order.

[a] Relatives without antithrombin deficiency.
[b] Age at recruitment.
[c] Classification uncertain, see Discussion.

pathogenic by PolyPhen-2, PhD-SNP (sequence and profile-based prediction) and MutPred2, with the exception of the p.(Lys146Glu), which was evaluated to be likely pathogenic by PolyPhen-2 and PhD-SNP, but benign by MutPred2. Physical location of the 7 single nucleotide variants in the antithrombin molecule is shown in Fig. 1.

Type I antithrombin deficiency was less common than type II (28% and 72%, respectively). Type I antithrombin deficiency was caused by deletions or a single nucleotide variant (p.(Ser214Phe)) in exon 4 (Tables 1 and 2). Variants causing type II HBS deficiency were all found in exons 2 and 3. One single nucleotide variant causing type II RS deficiency (p.(Ser426Leu)) was encoded by exon 7.

All single nucleotide variants were heterozygous, including a proposed compound heterozygous variant (two different mutated alleles in *SERPINC1*: p.(Arg45Trp) and p.(Pro73Leu), Table 1). Type of deficiency was determined by antithrombin antigen levels (type I or II distinction) and sequencing (known variants causing subgroups of type II deficiency).

The most common single nucleotide variant was c.218C > T, p.(Pro73Leu) (rs121909551), causing type II HBS antithrombin deficiency, observed in 41% of all carriers, and in 58% of the carriers of type II antithrombin deficiency. This single nucleotide variant was also observed in 0.14% of the ExAC population.

3.1. Type of antithrombin deficiency and venous thromboembolism

Sixteen of 46 carriers (35%) had experienced a venous thromboembolism at an age of 29 years in average (range: 16–60 years). A larger proportion of carriers of type I and type II RS than type II HBS deficiency experienced a first-time (60–69% versus 15%, $P = 0.001$; Table 2) and a recurrent venous thromboembolism (20–31% versus 0%, $P = 0.01$; Table 2). Compared to the carriers of type II HBS antithrombin deficiency, the carriers of type I and II RS deficiency had more first-degree relatives with venous thromboembolism, more severe thromboembolism and were more often referred due to having events (Table 2). None of the relatives without antithrombin deficiency have experienced a venous thromboembolism.

3.2. SERPINC1 variants and antithrombin activity

The carriers had an antithrombin activity of 56% in average. All but three carriers had an activity of 45–70%. The two siblings who proposedly were compound heterozygotes had an antithrombin activity of 22% and 24%, while a single carrier of a single nucleotide variant at the reactive site, had an activity of 80% at study entry (Table 1). However, the average antithrombin activity for the latter individual's five last measurements was 67%.

The relatives without antithrombin deficiency had an antithrombin activity of 109% in average (range: 97–120%).

3.3. Type II HBS antithrombin deficiency and heterozygosity for factor V Leiden

Heterozygosity for factor V Leiden was observed in 12% (three out of 25) of carriers of type II HBS deficiency. Only 16% (four out of 25) of carriers of type II HBS antithrombin deficiency experienced venous thromboembolism, and half of these carriers (two out of four) were heterozygous for factor V Leiden ($P = 0.06$). The four carriers with venous thromboembolism were not related: two of them had no relatives in the study, while other two had one first-degree relative each (factor V Leiden wildtype and no venous thromboembolism). Only one carrier who was heterozygous for factor V Leiden did not develop venous thromboembolism. This individual had the p.(Pro73Leu) single nucleotide variant and also had four uncomplicated pregnancies. Her four children were included in the study (two as carriers and two as relatives without antithrombin deficiency).

Table 2
Characteristics of participants according to type of antithrombin deficiency.

	Type I	Type II RS	Type II HBS	*P*-value
Total number of participants, N	13	5	27	NA
Age[a], mean (range) in years	44 (19–62)	35 (19–48)	46 (21–72)	0.72
Age at 1st event, mean (range) in years	25 (16–40)	23 (16–36)	42 (25–60)	0.11
Women, N (%)	8 (62)	4 (80)	16 (59)	0.82
1st degree relatives with venous thromboembolism, N (%)	10 (77)	4 (80)	15 (56)	0.38
Venous thromboembolism, N (%)	9 (69)	3 (60)	4 (15)	0.001
Deep vein thrombosis only, N (%)	6 (67)	1 (33)	4 (100)	0.54
Pulmonary embolism, N (%)	2 (22)	1 (33)	0 (0)	
Visceral thromboembolism, N (%)	1 (11)	1 (33)	0 (0)	
Reffered due to:				
Event, N (%)	7 (54)	3 (60)	8 (30)	0.37
Family history, N (%)	6 (46)	2 (40)	15 (56)	
Other, N (%)	0 (0)	0 (0)	4 (15)	
Recurrent venous thromboembolism, N (%)	4 (31)	1 (20)	0 (0)	0.01

Event = venous or arterial thromboembolism and/or obstetrical complications.
Family history = family history of thromboembolism or antithrombin deficiency.
RS = reactive site. HBS = heparin binding site. NA = not applicable.
P-value is for Fisher's exact test, except for age, where Cuzick's nonparametric test for trend was used.
[a] Age in years at recruitment.

4. Discussion

In this study of 46 carriers (from 23 families) of antithrombin deficiency, we detected causal variants in every single carrier, i.e. the variant detection rate was 100%. This is remarkable because we used the same approach as earlier studies of 163–188 carriers of antithrombin deficiency that only had a variant detection rate of approximately 80% [17,18].

All detected single nucleotide variants have been reported previously. The single nucleotide variant p.(Ser214Phe) caused type I deficiency, possibly due to a substitution of a small serine by a larger phenylalanine, that may have prevented correct α-helix F packing against β-sheet A [19]. The single nucleotide variant p.(Ser426Leu) caused type II RS deficiency, possibly due to a substitution of serine by the strongly apolar leucine, that may have made the reactive site less accessible for binding by thrombin [20].

Heparin and the closely related heparan sulfate bind with high affinity to the amino acids Arg45, Pro73, Arg79 and Lys146 at the HBS [21–23]. This exposes the reactive site, and increases the inhibitory activity of antithrombin by approximately 1000-fold [21]. All four residues are highly conserved among antithrombins from 13 vertebrate sequences known representing all five vertebrate families, thus illustrating their importance [21]. More than half of the carriers of type II deficiency were carriers of p.(Pro73Leu). A previous Danish study of 16 patients with antithrombin deficiency and a negative Denaturating High Performance Liquid Chromatography (DHPLC) variant screening, found this single nucleotide variant in half of the patients when sequencing was performed [24]. Also, p.(Pro73Leu) was one the most common single nucleotide variants detected in the ExAC database with a frequency of 0.14% in this population (URL: http://exac.broadinstitute.org/gene/ENSG00000117601 [August 2018]). Finally, in a Finnish study of 104 patients with type II antithrombin deficiency, this single nucleotide variant was observed in more than 85% of patients and families [25]. Deep vein thrombosis had a prevalence of 22% in the Finnish study, which is not so different from the 15% reported by our study. There seems to be a heterogeneity in geographic distribution of single nucleotide variants causing antithrombin deficiency. While p.(Pro73Leu) is a founder single nucleotide variant in Finland, and probably also common in Denmark, p.(Ala416Ser), a type II RS single nucleotide variant is the most common variant in The United Kingdom [4] and Spain [6] with a frequency of up to 0.2% of the general population. The latter single nucleotide variant was not detected in our study population, even though this single nucleotide variant has previously been detected in Denmark [24]. We observed p.(Ala175Asp) single nucleotide variant in one single carrier, who had not experienced a venous thromboembolism. This single nucleotide variant has previously only been reported in 2 individuals from one study [17]. It is located on strand 2A and partially buried under α-helix F, which would destabilize the molecule and might cause latency, suggesting that this might cause type II PE antithrombin deficiency. Additional in-vitro studies such as heparin affinity and progressive activity assays and analysis of plasma antithrombin by Western blot etc., are required to conclusively define the type of antithrombin deficiency. Unfortunately, we were unable to perform these analyses.

We confirmed the previous finding that venous thromboembolism was most common in type I, less common in type II RS, and least common in type II HBS antithrombin deficiency [17]. This is in agreement with the fact that homozygosity for type I antithrombin deficiency has not been reported, and is thought to be incompatible with life, while homozygosity for some type II HBS single nucleotide variants has been reported, and is associated with severe venous thromboembolism in childhood [26–28]. In this study, all the heterozygous carriers had half of the normal antithrombin activity, while the two siblings who proposedly were compound heterozygous had quarter of the normal antithrombin activity. The latter suggests that the siblings had inherited one single nucleotide variant on each allele. However, we do not know whether this is truly the case, and what role each single nucleotide variant had on the final anticoagulant activity. Neither sibling, an older brother and a younger sister, had experienced venous thromboembolism. However, the sister had a transitory cerebral ischemia at a young age, and is in treatment with warfarin. She was specifically not in treatment with oral contraceptives at the time of diagnosis due to her migraine diagnosis. However, she has had two spontaneous miscarriages, one in first and the other in second trimester, and one normal pregnancy with a minor placental thrombosis. According to the siblings, only their mother was diagnosed with antithrombin deficiency, and neither parent had experienced venous thromboembolism. We were not able to confirm this by medical records as neither parent participated in the study.

Only four (15%) of carriers of type II HBS deficiency experienced venous thromboembolism. Half of them were also heterozygous for factor V Leiden. This suggests that a combination of these two genetic risk factors has a synergistic effect on risk of venous thromboembolism. Indeed, only one carrier of both genetic risk factors was free from venous thromboembolism. Interestingly, this individual had four uncomplicated pregnancies, illustrating that genetic modifiers [29], or some other risk factors also play a role in developing venous thromboembolism. Our study was underpowered to answer the question

whether the combination of antithrombin type II HBS deficiency and factor V Leiden single nucleotide variant conferred a higher risk of venous thromboembolism than either risk factor alone. Heterozygosity for factor V Leiden was present in 12% of the carriers of type II HBS antithrombin deficiency, similar to 8% in the general population.

The strength of this study is that it is the largest Danish study systematically reporting *SERPINC1* variants causing hereditary antithrombin deficiency. Limitations include survival bias, selective non-response to the invitation, lack of information on diagnoses of arterial thrombosis, and lack of additional analyses for the characterisation of the type of antithrombin deficiency. Another possible limitation is that we only used a single coagulation factor Xa based method for measuring antithrombin activity. This method has been reported to overestimate antithrombin activity, especially in the presence of type II HBS deficiency such as p.(Pro73Leu) and p.(Arg79His) [8,24,30,31]. Despite the numerous functional assays available, no single assay is able to detect all the deficiencies [31]. This is further supported by studies reporting that among individuals with venous thromboembolism and normal antithrombin activity, 3–5% had variants causing hereditary antithrombin deficiency [32,33]. This suggests that true antithrombin deficiency is probably underestimated. Indeed, on a single occasion, we measured normal antithrombin activity (80%) in a single carrier of type II RS deficiency. Genetic testing can help our understanding of the underlying molecular pathomechanisms and could be considered for the purpose of 1) differentiating between acquired and inherited thrombophilia 2) in a diagnostic work-up of patients with unexplained venous thromboembolism and normal antithrombin activity, and 3) in first-degree relatives of individuals with a known variant.

In conclusion, in this study of 46 carriers of antithrombin deficiency, we achieved a variant detection rate of 100%. The majority of carriers had type II HBS deficiency, most often caused by the p.(Pro73Leu) single nucleotide variant, a Finnish founder single nucleotide variant. This and four other type II single nucleotide variants (Thr147Ala (rs2227606), Pro305His (rs549991084), Val30Glu (rs2227624) and Ala416Ser (rs121909548)), were detected in almost 1% of the ExAC population (which is 5 times the presently estimated prevalence in the general population), suggesting that the prevalence of type II HBS deficiency in the general population is probably underestimated. Future studies are needed to determine the prevalence of *SERPINC1* variants in the general population, and whether a combination of antithrombin type II HBS deficiency and other common genetic risk factors confers a higher risk of cardiovascular events (venous and arterial) than either risk factor alone.

Acknowledgement

First and foremost, we are deeply thankful to the participants for their willingness to participate.

We thank the staff at the Department of Clinical Biochemistry, Aarhus University Hospital for help with the recruitment process and laboratory analyses.

Funding

This research was supported by the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark. No other specific grants from funding agencies in the public, commercial, or not-for-profit sectors were received.

References

[1] J.C. Whisstock, R.N. Pike, L. Jin, R. Skinner, X.Y. Pei, R.W. Carrell, et al., Conformational changes in serpins: II. The mechanism of activation of antithrombin by heparin, J. Mol. Biol. 301 (2000) 1287–1305.
[2] J. Corral, M.E. de la Morena-Barrio, V. Vicente, The genetics of antithrombin, Thromb. Res. 169 (2018) 23–29.
[3] M.N.D. Di Minno, P. Ambrosino, W. Ageno, F. Rosendaal, G. Di Minno, F. Dentali, Natural anticoagulants deficiency and the risk of venous thromboembolism: a meta-analysis of observational studies, Thromb. Res. 135 (2015) 923–932.
[4] R.C. Tait, I.D. Walker, D.J. Perry, S.I.A.M. Islam, M.E. Daly, F. McCall, et al., Prevalence of antithrombin deficiency in the healthy population, Br. J. Haematol. 87 (1994) 106–112.
[5] P.S. Wells, M.A. Blajchman, P. Henderson, M.J. Wells, C. Demers, R. Bourque, et al., Prevalence of antithrombin deficiency in healthy blood donors: a cross-sectional study, Am. J. Hematol. 45 (1994) 321–324.
[6] J. Corral, D. Hernandez-Espinosa, J.M. Soria, R. Gonzalez-Conejero, A. Ordonez, J.R. Gonzalez-Porras, et al., Antithrombin Cambridge II (A384S): an underestimated genetic risk factor for venous thrombosis, Blood 109 (2007) 4258–4263.
[7] F.N. Croles, J. Borjas-Howard, K. Nasserinejad, F.W.G. Leebeek, K. Meijer, Risk of venous thrombosis in antithrombin deficiency: a systematic review and Bayesian meta-analysis, Semin. Thromb. Hemost. 44 (2018) 315–326, https://doi.org/10.1055/s-0038-1625983.
[8] C. Orlando, O. Heylen, W. Lissens, K. Jochmans, Antithrombin heparin binding site deficiency: a challenging diagnosis of a not so benign thrombophilia, Thromb. Res. 135 (2015) 1179–1185.
[9] H.H. van Boven, P.H. Reitsma, F.R. Rosendaal, T.A. Bayston, V. Chowdhury, K.A. Bauer, et al., Factor V Leiden (FV R506Q) in families with inherited antithrombin deficiency, Thromb. Haemost. 75 (1996) 417–421.
[10] K. Juul, A. Tybjaerg-Hansen, R. Steffensen, S. Kofoed, G. Jensen, B.G. Nordestgaard, Factor V Leiden: the Copenhagen City heart study and 2 meta-analyses, Blood 100 (2002) 3–10.
[11] B.F. Sode, K.H. Allin, M. Dahl, F. Gyntelberg, B.G. Nordestgaard, Risk of venous thromboembolism and myocardial infarction associated with factor V Leiden and prothrombin mutations and blood type, CMAJ 185 (2013) E229–E237.
[12] D.A. Lane, K. Auberger, H. Ireland, A.A. Roscher, S.L. Thein, Prenatal diagnosis in combined antithrombin and factor V gene mutation, Br. J. Haematol. 94 (1996) 753–755.
[13] D. Gemmati, M.L. Serino, S. Moratelli, R. Mari, G. Ballerini, G.L. Scapoli, Coexistence of antithrombin deficiency, factor V Leiden and hyperhomocysteinemia in a thrombotic family, Blood Coagul. Fibrinolysis 9 (1998) 173–176.
[14] C. Nielsen, J. Gram, Combined antithrombin deficiency and factor V Leiden in a Danish family, Ugeskr. Laeger 176 (2014) 25A.
[15] A. Pavlova, O. El-Maarri, B. Luxembourg, E. Lindhoff-Last, L. Kochhan, H.-D. Bruhn, et al., Detection of heterozygous large deletions in the antithrombin gene using multiplex polymerase chain reaction and denatured high performance liquid chromatography, Haematologica 91 (2006) 1264–1267.
[16] J.T. den Dunnen, R. Dalgleish, D.R. Maglott, R.K. Hart, M.S. Greenblatt, J. McGowan-Jordan, et al., HGVS recommendations for the description of sequence variants: 2016 update, Hum. Mutat. 37 (2016) 564–569.
[17] B. Luxembourg, D. Delev, C. Geisen, M. Spannagl, M. Krause, W. Miesbach, et al., Molecular basis of antithrombin deficiency, Thromb. Haemost. 105 (2011) 635–646.
[18] M. Caspers, A. Pavlova, J. Driesen, U. Harbrecht, R. Klamroth, J. Kadar, et al., Deficiencies of antithrombin, protein C and protein S - practical experience in genetic analysis of a large patient cohort, Thromb. Haemost. 108 (2012) 247–257.
[19] V. Picard, A. Bura, J. Emmerich, M. Alhenc-Gelas, C. Biron, L.L. Houbouyan-Reveillard, et al., Molecular bases of antithrombin deficiency in French families: identification of seven novel mutations in the antithrombin gene, Br. J. Haematol. 110 (2000) 731–734.
[20] A.W. Stephens, B.S. Thalley, C.H. Hirs, Antithrombin-III Denver, a reactive site variant, J. Biol. Chem. 262 (1987) 1044–1048.
[21] M. Backovic, P.G.W. Gettins, Insight into residues critical for antithrombin function from analysis of an expanded database of sequences that includes frog, turtle, and ostrich antithrombins, J. Proteome Res. 1 (2002) 367–373.
[22] A. Mushunje, A. Zhou, J.A. Huntington, J. Conard, R.W. Carrell, Antithrombin 'DREUX' (Lys 114Glu): a variant with complete loss of heparin affinity, Thromb. Haemost. 88 (2002) 436–443.
[23] V. Picard, S. Susen, S. Bellucci, M. Aiach, M. Alhenc-Gelas, Two new antithrombin variants support a role for K114 and R13 in heparin binding, J. Thromb. Haemost. 1 (2003) 386–387.
[24] S. Feddersen, M. Nybo, Discordant diagnoses obtained by different approaches in antithrombin mutation analysis, Clin. Biochem. 47 (2014) 1337–1340.
[25] M. Puurunen, P. Salo, S. Engelbarth, K. Javela, M. Perola, Type II antithrombin deficiency caused by a founder mutation Pro73Leu in the Finnish population: clinical picture, J. Thromb. Haemost. 11 (2013) 1844–1849.
[26] A. Bauters, C. Zawadzki, A. Bura, C. Théry, A. Watel, D. Subtil, et al., Homozygous variant of antithrombin with lack of affinity for heparin: management of severe thrombotic complications associated with intrauterine fetal demise, Blood Coagul. Fibrinolysis 7 (1996) 705–710.
[27] V. Picard, M.-D. Dautzenberg, B.O. Villoutreix, G. Orliaguet, M. Alhenc-Gelas, M. Aiach, Antithrombin Phe229Leu: a new homozygous variant leading to spontaneous antithrombin polymerization in vivo associated with severe childhood thrombosis, Blood 102 (2003) 919–925.
[28] R. Gindele, Z. Oláh, P. Ilonczai, M. Speker, Á. Udvari, A. Selmeczi, et al., Founder effect is responsible for the p.Leu131Phe heparin-binding-site antithrombin mutation common in Hungary: phenotype analysis in a large cohort, J. Thromb. Haemost. 14 (2016) 704–715.
[29] M. Kousi, N. Katsanis, Genetic modifiers and oligogenic inheritance, Cold Spring Harb. Perspect. Med. 5 (2015) a017145, https://doi.org/10.1101/cshperspect.a017145.
[30] J.S. Ungerstedt, Discrepancy between antithrombin activity methods revealed in Antithrombin Stockholm: do factor Xa-based methods overestimate antithrombin activity in some patients? Blood 99 (2002) 2271–2272.

[31] K. Javela, S. Engelbarth, L. Hiltunen, P. Mustonen, M. Puurunen, Great discrepancy in antithrombin activity measured using five commercially available functional assays, Thromb. Res. (2013) 132–137.
[32] R. Fischer, U.J. Sachs, K.S. Heidinger, D. Eisenburger, B. Kemkes-Matthes, Prevalence of hereditary antithrombin mutations is higher than estimated in patients with thrombotic events, Blood Coagul. Fibrinolysis 24 (2013) 444–448.
[33] W. Zeng, L. Tang, X.R. Jian, Y.Q. Li, T. Guo, Q.Y. Wang, et al., Genetic analysis should be included in clinical practice when screening for antithrombin deficiency, Thromb. Haemost. 113 (2) (2015) 262–271, https://doi.org/10.1160/TH14-05-0446.