

# SERPINC1 gene mutations in antithrombin deficiency

René Mulder,<sup>1</sup>  F. Nanne Croles,<sup>2,3</sup>  
André B. Mulder,<sup>1</sup> James A.  
Huntington,<sup>4</sup> Karina Meijer<sup>2</sup> and  
Michaël V. Lukens<sup>1</sup>

<sup>1</sup>Department of Laboratory Medicine, University Medical Centre Groningen, <sup>2</sup>Division of Haemostasis and Thrombosis, Department of Haematology, University Medical Centre Groningen, Groningen, <sup>3</sup>Department of Haematology, Erasmus University Medical Centre, Rotterdam, the Netherlands and <sup>4</sup>Department of Haematology, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

Received 4 October 2016; accepted for publication 16 January 2017

Correspondence: Dr. René Mulder, Laboratory Medicine, University Medical Centre Groningen, Hanzplein 19700 RB Groningen, The Netherlands  
E-mail: r.mulder01@umcg.nl

## Abstract

Existing evidence suggests that in most cases antithrombin deficiency can be explained by mutations in its gene, *SERPINC1*. We investigated the molecular background of antithrombin deficiency in a single centre family cohort study. We included a total of 21 families comprising 15 original probands and sixty-six relatives, 6 of who were surrogate probands for the genetic analysis. Antithrombin activity and antigen levels were measured. The heparin-antithrombin binding ratio assay was used to distinguish between the different subtypes of type II antithrombin deficiency. *SERPINC1* mutations were detected by direct sequencing of all 7 exons and regulatory regions, and multiplex ligation-dependent probe amplification. Eighty-six per cent of the families had a detrimental *SERPINC1* gene mutation that segregated in the family. We detected 13 different *SERPINC1* gene mutations of which 5 were novel. Among all these mutations, 44% was associated with type I deficiency, whereas the remainder was associated with type II heparin binding site (11%), type II pleiotropic effect (33%), type II reactive site (6%) or had the antithrombin Cambridge II mutation (6%). The current study reports several novel *SERPINC1* mutations, thereby adding to our knowledge of the molecular background of antithrombin deficiency. Finally, our results point out the importance of future research outside the conventional *SERPINC1* gene approach.

**Keywords:** *SERPINC1*, antithrombin, antithrombin deficiency, sequencing, MLPA.

Antithrombin is a member of the serine proteinase inhibitor (serpin) superfamily, which has a highly conserved structure, with three  $\beta$ -sheets (A–C) and eight to nine  $\alpha$ -helices (A–I) (law *et al*, 2006). Antithrombin is the major physiological inhibitor of coagulation but also exhibits anti-inflammatory properties, thereby accentuating the crosstalk between both pathways (Levy *et al*, 2016).

The inhibitory function of antithrombin is mediated principally through targeting thrombin, factor Xa and factor IXa. However, the inhibitory function of antithrombin is remarkably slow due to its repressed reactivity state. Heparin or heparan sulfate-type glycosaminoglycan cofactors transform antithrombin into a fast inhibitor by relieving this repression. This explains the therapeutic use of heparin as an anticoagulant.

The clinical significance of antithrombin function is clearly demonstrated by the fact that its deficiency (Online Mendelian Inheritance in Man (OMIM) reference 107300) is associated with a strong risk of venous thrombosis. Antithrombin deficiency can be classified into type I

(quantitative defect) and type II (qualitative defect). Type I is characterized by a parallel reduction of both the antigen levels and functional activity. Type II is characterized by low activity levels and normal antithrombin antigen levels. Depending on the location of the mutations in the gene of antithrombin, *SERPINC1*, type II deficiency can be further subdivided in type II RS (reactive site), type II HBS (heparin binding site) and type II PE (pleiotropic effect) (Lane *et al*, 1997). Noteworthy, the p.Ala416Ser (antithrombin Cambridge II) is converted into a substrate in the presence of heparin (Mushunje *et al*, 2003). Although it is in the reactive centre loop, we cannot call it an RS mutant. Instead this mutation forms a unique class among the type II mutations.

Antithrombin deficiency is rare: the prevalence in healthy blood donors was found to be between 1:500 and 1:645 (Tait *et al*, 1994; Wells *et al*, 1994; Sakata *et al*, 2004), whereas in patients with venous thrombosis the prevalence was 1–5% (Heijboer *et al*, 1990; De Stefano *et al*, 2006; Rossi *et al*, 2008; Di Minno *et al*, 2014).

*SERPINC1* is located on chromosome 1q23-25 (Bock *et al*, 1985), contains seven exons and six introns, and spans 13 578 bp of genomic DNA. To date, more than 250 *SERPINC1* gene mutations have been reported to be associated with antithrombin deficiency [http://www.hgmd.cf.ac.uk/ac/; https://www1.imperial.ac.uk/departments/medicine/divisions/experimentalmedicine/haematology/coag/antithrombin/]. The mutation profile of the *SERPINC1* gene is heterogeneous, being typically comprised of point mutations, splice site variants and small insertion/deletion mutations, whereas gross rearrangements account for only a small proportion.

In this study, we investigated the molecular background of antithrombin deficiency in a single centre family cohort study.

## Material and methods

### Patients

The subjects in this single centre family cohort study were patients with previously diagnosed hereditary antithrombin deficiency or their first-degree relatives. Our study enrolled adult patients ( $\geq 18$  years) after written informed consent, between January 2013 and January 2015. Some of the probands were included in a previous cohort study (Brouwer *et al*, 2006; Mahmoodi *et al*, 2010). They largely stem from referrals to our tertiary centre for thrombophilia screening because of venous or arterial thrombotic events. First-degree relatives of probands were identified by pedigree analysis.

### Blood collection

Venous blood samples were anticoagulated with a 1:10 volume of 0.109 mol/l trisodium citrate. Platelet-poor plasma was prepared by centrifugation at 2500 g for 15 min. The samples were then aliquoted and immediately frozen at  $-80^{\circ}\text{C}$  until use, and analysed after rapid thawing at  $37^{\circ}\text{C}$ . Genomic DNA was obtained from EDTA samples using the Qiacube<sup>®</sup> system (QIAGEN, Hilden, Germany).

### Antithrombin assays

Antithrombin activity measurements were performed with an assay based on anti-FXa inhibition (INNOVANCE<sup>®</sup> Antithrombin assay; Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) (normal levels  $>80\%$ ). Antithrombin antigen levels were measured with an enzyme-linked immunosorbent assay (ELISA) with reagents obtained from Affinity Biologicals (Ancaster, ON, CANADA) (normal levels  $>80\%$ ). For the heparin-antithrombin binding (HAB) ratio we adopted the assay of Moore *et al* (2015). This assay quantifies the heparin-binding capacity of antithrombin by deriving a ratio of antithrombin activities generated from short (30 s) and prolonged incubation times (290 s) with heparin. A reduced HAB ratio ( $<0.8$ ) distinguishes type II HBS antithrombin deficiency from the other subtypes.

### Additional thrombophilic assays

Total protein S antigen levels were measured with an ELISA using the Asserachrom Protein S kit (Diagnostica Stago, Asnières sur Seine, France). Free protein S antigen levels were measured with a latex-based test (HemosIL; Instrumentation Laboratory Spa, Milan, Italy). Protein C antigen levels were measured with an ELISA using the Asserachrom Protein C kit from Diagnostica Stago. Protein C activity levels were measured with the Berichrom protein C test from Siemens. The presence of *F5* R506Q (Factor V Leiden) and *F2* G20210A (prothrombin mutation) was tested using the Cepheid Xpert<sup>®</sup> FII & FV on the Cepheid GeneXpert System (Cepheid, Sunnyvale, CA, USA).

### Genomic DNA amplification and sequence analysis of *cursief*

Direct sequencing analysis of all 7 exons and flanking introns of *SERPINC1* was performed to detect sequence variations in the (surrogate) probands. Additionally, multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA probemix P227-B2 *SERPINC1* (MRC-Holland, Amsterdam, The Netherlands; lot B2-0314), and direct sequencing of the promoter region (1500 bp upstream of exon 1) (de la Morena-Barrio *et al*, 2012) was performed if no sequence variation was detected by direct sequencing of all 7 exons and flanking introns. If a sequence variation was found in the proband, all relatives were tested for that specific variant. Primer sequences and PCR conditions can be obtained from the authors upon request.

### In silico prediction tools

In silico tools for pathogenicity prediction of exonic *SERPINC1* variants were chosen based on having the highest performance characteristics in a study by Luxembourg *et al* (2015). These tools included MutPred (Li *et al*, 2009), PhD-SNP (Capriotti *et al*, 2006), and PolyPhen-2 HumDiv (Adzhubei *et al*, 2010). In addition, we used MutationTaster (Schwarz *et al*, 2014) because it enabled us to perform predictions of pathogenicity at base pair level. MutationTaster also uses a locally installed third party prediction program, called NNSplice (Reese *et al*, 1997) that analyses possible changes in splice sites. This program, together with Human Splicing Finder (HSF) V3 (Desmet *et al*, 2009), were used to analyse possible changes in splice sites.

### Protein sequence alignment of antithrombin from vertebrates

To determine the degree of conservation we used the results of the protein sequence alignment of antithrombin from 45 vertebrate genomes (Kumar *et al*, 2013).

### Structural visualization

For structural visualization we used the native structure of  $\alpha$ -antithrombin (1E05; McCoy *et al*, 2003).

### Nomenclature

Mutalyzer 2.0.22 (<https://www.mutalyzer.nl/>) was used to check the descriptions of each *SERPINC1* sequence variant according to the standard Human Genome Sequence Variation Society (HGVS) nomenclature [<http://www.hgvs.org/mutnomen>] of the UK Association for Clinical Genetic Science and the American College for Medical Genetics [Wildeman *et al*, 2008; Richards *et al*, 2015]. Reference sequences used were NP\_000479.1, NM\_000488.3 (cDNA) and NG\_012462.1 (genomic). For convenience, the mature amino acid numbering (minus the 32 amino acid signal peptide) is also shown in parenthesis.

## Results

A total of 21 families were included, of which the original proband was available for 15 families. For the other 6 families, a family member with previously diagnosed antithrombin deficiency and a history of VTE was used as surrogate proband for genetic testing. Sixty-six relatives were available for testing. No deficiency of protein C or S was found. Families H and Q had surrogate probands that carry the *F5* R506Q. Furthermore, 3 members of Family I, including the proband carry the *F2* G20210A.

### SERPINC1 gene analysis and in silico prediction

Eighteen out of 21 families had a detrimental *SERPINC1* gene mutation that was only found in antithrombin-deficient individuals (Table I). Three families (D, P and U) remained mutation-negative after application of additional mutation screening (MLPA and promotor region). In total, we identified 13 different *SERPINC1* gene mutations, with 8 missense mutations, 3 deletions, 1 insertion, and 1 splice site variant (Table I). This mutation profile is in agreement with previous reports [<http://www.hgmd.cf.ac.uk/ac/>; <https://www1.imperia.lac.uk/departamentofmedicine/divisions/experimentalmedicine/haematology/coag/antithrombin/>].

Eight of these mutations have been reported previously, whereas 5 were novel (Fig 1). One of the novel mutations occurred in 3 probands/families. Upon further questioning, we learned that two families were related.

All *SERPINC1* gene mutations were found in a heterozygous state. All in silico predictions confirmed the pathogenicity of all mutations, except for p.Tyr190Cys (158), p.Val327Glyfs\*16 (285), and p.Glu345del (303). In case of p.Tyr190Cys (158), all in silico prediction tools, except for MutPred, predicted this mutation to be pathogenic. Using MutPred, p.Tyr190Cys (158) had a score of 0.31, which is

not high enough (0.50) for it to be designated as a pathogenic mutation. For the novel p.Val327Glyfs\*16 (285) we retrieved a prediction with only MutationTaster, which was 'disease causing'. Interestingly, MutationTaster classified p.Glu345del (303) as being a polymorphism, which is in contrast with Chowdhury *et al* (1993), who clearly stated that it is a known disease causing mutation at this position.

Based on the protein sequence alignment of antithrombin from 45 vertebrate genomes, all missense mutations were shown to affect a site that was highly conserved (70%), whereas three out of 8 missense mutations affected a site that was fully conserved (100%) (Kumar *et al*, 2013). These results suggest a strong negative effect on the overall structure of antithrombin.

### Genotype-phenotype associations

Overall, 52% of probands had a type I, and 48% had a type II antithrombin deficiency. All three mutation-negative probands had a type I antithrombin deficiency with a mean antithrombin antigen of 51% (range 42–63%), and a mean antithrombin activity of 60% (range 50–69%). The mean HAB ratio in these probands was 0.93, which indicates that the interaction between antithrombin and heparin is normal (>0.8). Of the families where a causative mutation was detected, 44% had a type I antithrombin deficiency and 56% had a type II antithrombin deficiency. In families with a type I antithrombin deficiency, the mean antithrombin antigen was 53% (range 41–73%), and the mean antithrombin activity was 54% (range 44–72%).

The families with a type II antithrombin deficiency had a mean antithrombin antigen of 88% (range 60–136%), and a mean antithrombin activity of 60% (range 49–72%). The type II could be further subdivided on basis of the type of mutation and HAB ratio, where a HAB ratio below 0.8 indicates a type II HBS while a HAB ratio above 0.8 indicates either type I or type II (RS of PE). We found the following subtypes: type II PE, (33%), type II RS (6%), type II HBS (11%), and those carrying the antithrombin Cambridge II mutation (6%). In the HBS patients the mean HAB ratio was 0.73 (range 0.67–0.77).

## Discussion

Antithrombin deficiency (OMIM 107300) is associated with an increased risk of venous thrombosis. Therefore, unravelling the molecular background of this thrombophilic disorder is important to give more insight in to the risk of developing venous thrombosis. Our study detected a causal mutation in 86% of the families with antithrombin deficiency. These results are in agreement with recent studies by Luxembourg *et al* (2011) and Caspers *et al* (2012), who found a candidate gene mutation in 80% and 84% of all patients, respectively.

The results of the tests remained uninformative in three type I families (D, P and U). Linkage studies would have

Table I. Characteristics of 21 antithrombin deficient families.

Family	Sex	Age at VTE (years)	AT:act (%)	AT:ag (%)	HAB ratio	Type	Location	Cytogenetic Nomenclature	Phenotypic Nomenclature	Reference	In silico prediction*	Tested family members	AT deficient/ mutation	Normal/ mutation
A	M	17	49	70	0.97	II PE	Exon 3	c.536T>G	p.Phe179Cys	None	Pathogenic	1	1/1	0/0
B	F	24	56	67	0.93	I	Exon 5	c.857A>C	p.Gln286Pro	None	Pathogenic	8	8/8	0/0
C	M	No VTE	72	96	0.77	II HBS**	Exon 2	c.218C>T	p.Pro73Leu	Tran et al (1980)	Pathogenic	4	2/2	2/0
D	M	70	61	42	0.91	I	No mutation detected.				NA	2	NA	NA
E	F	27	49	46	0.90	I	Exon 5	c.857A>C	p.Gln286Pro	None	Pathogenic	4	3/3	1/0
F	M	No VTE	55	82	0.95	II PE	Exon 2	c.377C>G	p.Ala126Gly	Luxembourg et al (2011)	Pathogenic	4	3/3	1/0
G	F	16	59	93	0.94	II PE	Exon 3	c.569A>G	p.Tyr190Cys	Millar et al (1994)	Pathogenic†	7	4/4	3/0
H	M	17	47	41	0.88	I	Intron 4	c.763-1G>A		None	Pathogenic	4	2/2	2/0
I	F	40	53	77	0.93	II PE	Exon 2	c.377C>G	p.Ala126Gly	Luxembourg et al (2011)	Pathogenic	4	4/4	0/0
J	F	66	61	58	0.93	I	Exon 5	c.979dup	p.Val327Glyfs*16	None	Pathogenic‡	5	2/2	3/0
K	F	No VTE	72	46	0.94	I	Exon 2	c.337_338delCT	p.Leu113Efs*15	Chowdhury et al (1993)	Pathogenic‡	1	1/1	0/0
L	M	29	68	99	0.83	II PE	Exon 4	c.749C>T	p.Thr250Ile	None	Pathogenic	2	2/2	0/0
M	M	41	67	53	0.93	I	Exon 5	c.1056del	p.Met352Ilefs*12	van Boven et al (1994)	Pathogenic‡	7	4/4	3/0
N	F	17	58	73	0.95	I	Exon 5	c.1033_1035del	p.Glu345del	Chowdhury et al (1993)	Pathogenic§	2	2/2	0/0
O	F	21	51	103	1.01	II¶	Exon 7	c.1246G>T	p.Ala416Ser	Perry et al (1991)	Pathogenic	2	1/1	1/0
P	F	26	69	48	0.96	I	No mutation detected.				NA	2	NA	NA
Q	F	25	71	128	0.72	II HBS**	Exon 2	c.218C>T	p.Pro73Leu	Tran et al (1980)	Pathogenic	4	2/2	2/0
R	M	4	70	136	0.90	II RS	Exon 7	c.1274G>A	p.Arg425His	Erdjument et al (1988)	Pathogenic	3	1/1	2/0
S	F	62	49	49	0.93	I	Exon 5	c.857A>C	p.Gln286Pro	None	Pathogenic	6	3/3	3/0
T	F	27	56	76	0.89	II PE	Exon 3	c.569A>G	p.Tyr190Cys	Millar et al (1994)	Pathogenic†	5	2/2	3/0
U	M	No VTE	53	58	0.88	I	No mutation detected.				NA	2	NA	NA

AT, antithrombin; AT:act, antithrombin activity; AT:ag, antithrombin antigen; F, female; HAB, heparin-antithrombin binding; HBS, heparin binding site; M, male; NA, not applicable; PE, pleiotropic effect; RS, reactive site; VTE, venous thromboembolism.

\*Results of in silico prediction for pathogenicity was combined. For more details on type of tools used see methods section.

†All in silico prediction tools, except for MutPred, predicted this mutation to be pathogenic.

‡We retrieved a prediction with only Mutationtaster, which was 'disease causing'.

§We retrieved a prediction with only Mutationtaster, which was 'polymorphism'.

¶This mutation is called antithrombin Cambridge II and is known to form an unique class among the type II mutations (Mushunje et al, 2003).

\*\*Questionable association (see text).

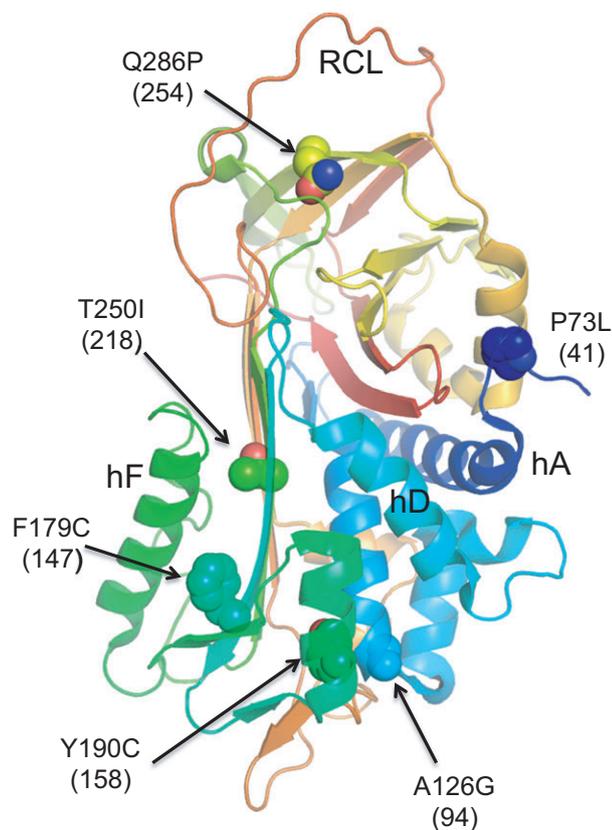


Fig 1. Structure of native antithrombin showing position of missense mutations. Native alpha antithrombin (1E05) is shown as a ribbon diagram, coloured in rainbow, from N-terminus (blue) to C-terminus red. It is shown in the standard orientation (reactive centre loop [RCL] on top), but turned to the side to better visualize the sites of the mutations. Helices (h) A, D and F are indicated. For convenience, we also included in the text the mature amino acid numbering (minus the 32 amino acid signal peptide) in parenthesis.

increased our chance of finding the molecular defect in these three families, but were not part of this study. Nonetheless, these three families did include first- and second-degree relatives with antithrombin type I deficiency. This makes it likely that this phenotype is caused by the genetic defect found in these families. Despite the absence of linkage data, other potential causes for antithrombin deficiency in these patients may be found outside the direct molecular context of *SERPINC1* gene regulation, such as microRNAs (Teruel-Montoya *et al*, 2015) or the antithrombin-modulating gene *LARGE1* (de la Morena-Barrio *et al*, 2013). Recently, de la Morena-Barrio *et al* (2016) identified hypoglycosylation, not only of antithrombin but also of other N-glycoproteins in 8 out of 30 antithrombin-deficient cases without alterations in *SERPINC1*. Moreover, evidence is mounting with regard to the association between DNA methylation and thrombophilic risk factors (El-Maarri *et al*, 2007; Aissi *et al*, 2014; Dick *et al*, 2014).

The novel mutations included 3 missense mutations, 1 splice site variant and 1 insertion. The novel missense

mutation p.Phe179Cys (147) was identified in 1 family (Family A). This mutation is on s2A under helix F and will probably result in rapid conversion from native to latent. This would explain the relatively normal antigen level and low activity. Another novel missense mutation p.Thr250Ile (218) was also found in only 1 family (Family L). We termed this mutation as being novel, because there was no published clinical data on this variant. The primary submitter was NHLBI Exome Sequencing Project (ESP). The minor allele frequency was  $A = 0.00021978$ . This mutation is on s3A and is underneath hF and the hF-loop. It should increase the rate of latent conversion. Both mutations are expected to allow correct folding to native, but to increase the rate of conversion to latent. This seems similar to the wobble/wobble mutations described by Beauchamp *et al* (1998), and fully explains the discrepancy between antigen level and activity. We therefore termed these mutations as type II PE.

The novel missense mutation, p.Gln286Pro (254), was associated with type I antithrombin deficiency. Its position is consistent with induced polymerisation via the C-terminal mechanism, resulting in a secretion defect, with similar low antigen and activity levels. Noteworthy, another mutation at p.Gln286 (p.Gln286His) has been reported in dbSNP (rs139463995). This mutation has an allele frequency of  $8.247e-06$  [<http://exac.broadinstitute.org/>]. Furthermore, in silico prediction tools predicted this mutation to be pathogenic. Taken together, these results suggest that a mutation at this site is rare and unfavourable.

We identified 1 novel splice site variant at the acceptor site of exon 4. This variant was found in 1 family (Family H). Using NNSplice (Reese *et al*, 1997) and Human Splicing Finder (HSF) V3 (Capriotti *et al*, 2006), this variant was predicted to have a negative effect on splicing. This variant was associated with type I antithrombin deficiency. Such an association has been described before (Jochmans *et al*, 1994).

The final novel mutation p.Val327Glyfs\*16 was detected in exon 5 in 1 family (Family J). The apparent conformational sensitivity of serpins suggests that this insertion would never tolerate secretion. This is in agreement with the type I deficiency status.

In all families the mutations were only found in antithrombin-deficient individuals. However, for families A and K only 1 individual was available for testing (Table I). For families O and R, all tested family members, beside the proband, were non-deficient but did not carry a *SERPINC1* mutation (Table I). Not enough family members were tested in these four families to be sure that the molecular defect is disease causing. Three out of four *SERPINC1* mutations have been described previously (Erdjument *et al*, 1988; Perry *et al*, 1991; Chowdhury *et al*, 1993).

In this study, almost half of the *SERPINC1* mutations were associated with type I antithrombin deficiency, whereas the other half was associated with a type II antithrombin deficiency. In general, these associations do not deviate from the literature. However, in the study of Luxembourg *et al*

(2011) p.Ala126Gly (94) was associated with a type I phenotype, whereas our results led us to conclude that it is clearly a type II. There might be some folding defect leading to slightly normal antigen levels (82%), but the activity is clearly decreased (50%). The position of the mutation, highlighted in Fig 1, would also probably result in conversion to latent. Furthermore, there are some discrepancies between phenotype and *in silico* predictions of some type II mutations. For instance, on the basis of antithrombin levels and HAB ratio, p.Pro73Leu (Erdjument *et al*, 1988) can be classified as being a type II HBS. However, structural assessment of this mutation may question the association with type II, let alone with being type II HBS. The location is the flexible N-terminus, and although this is in position to affect the binding of heparin, there is no structural reason why it should do so. Further research is required to determine how the Pro to Leu substitution will affect heparin binding, hence, support the association with type II HBS phenotype. Additionally, p.Tyr190Cys (158) is on helix E and underlies sheet A. It would be predicted to result in a rapid conversion to latent. In the literature the reported association with antithrombin deficiency was said to be controversial for p.Tyr190Cys mutation as it did not cosegregate with the phenotype in the pedigrees from a previous study (Millar *et al*, 1994). However, our study showed a complete segregation and strong association with a type II. Based on the structural prediction we designated this mutation as type II PE.

Finally, in our study, the individual carrying the Cambridge II mutation had low antithrombin activity, which is in line with what would be expected using full-length heparin in the activity assay (Mushunje *et al*, 2003).

## References

- Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S. & Sunyaev, S.R. (2010) A method and server for predicting damaging missense mutations. *Nature Methods*, **7**, 248–249.
- Aïssi, D., Dennis, J., Ladouceur, M., Truong, V., Zwingerman, N., Rocanin-Arjo, A., Germain, M., Paton, T.A., Morange, P.E., Gagnon, F. & Tréguët, D.A. (2014) Genome-wide investigation of DNA methylation marks associated with FV Leiden mutation. *PLoS ONE*, **9**, e108087.
- Beauchamp, N.J., Pike, R.N., Daly, M., Butler, L., Makris, M., Dafforn, T.R., Zhou, A., Fitton, H.L., Preston, F.E., Peake, I.R. & Carrell, R.W. (1998) Antithrombins Wibble and Wobble (T85M/K): archetypal conformational diseases with *in vivo* latent-transition, thrombosis, and heparin activation. *Blood*, **92**, 2696–2706.
- Bock, S.C., Harris, J.F., Balazs, I. & Trent, J.M. (1985) Assignment of the human antithrombin III structural gene to chromosome 1q23-25. *Cytogenetics and Cell Genetics*, **39**, 67–69.
- van Boven, H.H., Olds, R.J., Thein, S.L., Reitsma, P.H., Lane, D.A., Briët, E., Vandenbroucke, J.P. & Rosendaal, F.R. (1994) Hereditary antithrombin deficiency: heterogeneity of the molecular basis and mortality in Dutch families. *Blood*, **84**, 4209–4213.
- Brouwer, J.L., Veeger, N.J., Kluijn-Nelemans, H.C. & van der Meer, J. (2006) The pathogenesis of venous thromboembolism: evidence for multiple interrelated causes. *Annals of Internal Medicine*, **145**, 807–815.
- Capriotti, E., Calabrese, R. & Casadio, R. (2006) Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. *Bioinformatics*, **22**, 2729–2734.
- Caspers, M., Pavlova, A., Driesen, J., Harbrecht, U., Klamroth, R., Kadar, J., Fischer, R., Kemkes-Matthes, B. & Oldenburg, J. (2012) Deficiencies of antithrombin, protein C and protein S - practical experience in genetic analysis of a large patient cohort. *Thrombosis and Haemostasis*, **108**, 247–257.
- Chowdhury, V., Olds, R.J., Lane, D.A., Conard, J., Pabinger, I., Ryan, K., Bauer, K.A., Bhavnani, M., Abildgaard, U., Finazzi, G., Castaman, G., Mannucci, P.M. & Thein, S.L. (1993) Identification of nine novel mutations in type I antithrombin deficiency by heteroduplex screening. *British Journal of Haematology*, **84**, 656–661.
- De Stefano, V., Simioni, P., Rossi, E., Tormene, D., Za, T., Pagnan, A. & Leone, G. (2006) The risk of recurrent venous thromboembolism in patients with inherited deficiency of natural anticoagulants antithrombin, protein C and protein S. *Haematologica*, **91**, 695–698.
- Desmet, F.O., Hamroun, D., Lalande, M., Collod-Bérout, G., Claustres, M. & Bérout, C. (2009) Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Research*, **37**, e67.
- Di Minno, M.N., Dentali, F., Lupoli, R. & Ageno, W. (2014) Mild antithrombin deficiency and risk of recurrent venous thromboembolism: a prospective cohort study. *Circulation*, **129**, 497–503.
- Dick, K.J., Nelson, C.P., Tsaprouni, L., Sandling, J.K., Aïssi, D., Wahl, S., Meduri, E., Morange, P.E., Gagnon, F., Grallert, H., Waldenberger, M., Peters, A., Erdmann, J., Hengstenberg, C., Cambien, F., Goodall, A.H., Ouwehand, W.H., Schunkert, H., Thompson, J.R., Spector, T.D., Gieger, C., Tréguët, D.A., Deloukas, P. &

## Acknowledgements

We would like to acknowledge Dr Gary Moore and Ms Naomi de Jager (Viapath Haemostasis & Thrombosis Laboratories at Guy's & St. Thomas' Hospitals, London, UK) for providing the assay settings for the heparin-antithrombin binding assay. Finally, we want to thank Krista A. Kooi BSc (Department of Genetics, University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands) for her technical support with the MLPA. This study was partly funded by an unrestricted grant from Bayer.

## Author contribution

RM, FNC, ABM, KM and MVL performed the research. RM, FNC, ABM, KM and MVL designed the research study. RM, FNC, ABM, JAH, KM and MVL analysed the data. RM, FNC, ABM, JAH, KM and MVL wrote the paper.

- Samani, N.J. (2014) DNA methylation and body-mass index: a genome-wide analysis. *Lancet*, **383**, 1990–1998.
- El-Maari, O., Becker, T., Junen, J., Manzoor, S.S., Diaz-Lacava, A., Schwaab, R., Wienker, T. & Oldenburg, J. (2007) Gender specific differences in levels of DNA methylation at selected loci from human total blood: a tendency toward higher methylation levels in males. *Human Genetics*, **122**, 505–514.
- Erdjument, H., Lane, D.A., Panico, M., Di Marzo, V. & Morris, H.R. (1988) Single amino acid substitutions in the reactive site of antithrombin leading to thrombosis. Congenital substitution of arginine 393 to cysteine in antithrombin Northwick Park and to histidine in antithrombin Glasgow. *The Journal of Biological Chemistry*, **263**, 5589–5593.
- Heijboer, H., Brandjes, D.P., Büller, H.R., Sturk, A. & ten Cate, J.W. (1990) Deficiencies of coagulation-inhibiting and fibrinolytic proteins in outpatients with deep-vein thrombosis. *The New England Journal of Medicine*, **323**, 1512–1516.
- Jochmans, K., Lissens, W., Yin, T., Michiels, J.J., van der Luit, L., Peerlinck, K., De Waele, M. & Liebaers, I. (1994) Molecular basis for type 1 antithrombin deficiency: identification of two novel point mutations and evidence for a *de novo* splice site mutation. *Blood*, **84**, 3742–3748.
- Kumar, A., Bhandari, A., Sarde, S.J. & Goswami, C. (2013) Sequence, phylogenetic and variant analyses of antithrombin III. *Biochemical and Biophysical Research Communications*, **440**, 714–724.
- Lane, D.A., Bayston, T., Olds, R.J., Fitches, A.C., Cooper, D.N., Millar, D.S., Jochmans, K., Perry, D.J., Okajima, K., Thein, S.L. & Emmerich, J. (1997) Antithrombin mutation database: 2nd (1997) update. For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thrombosis and Haemostasis*, **77**, 197–211.
- Law, R.H., Zhang, Q., McGowan, S., Buckle, A.M., Silverman, G.A., Wong, W., Rosado, C.J., Langendorf, C.G., Pike, R.N., Bird, P.I. & Whistock, J.C. (2006) An overview of the serpin superfamily. *Genome Biology*, **7**, 216.
- Levy, J.H., Sniecinski, R.M., Welsby, I.J. & Levi, M. (2016) Antithrombin: anti-inflammatory properties and clinical applications. *Thrombosis and Haemostasis*, **115**, 712–728.
- Li, B., Krishnan, V.G., Mort, M.E., Xin, F., Kamati, K.K., Cooper, D.N., Mooney, S.D. & Radivojac, P. (2009) Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics*, **25**, 2744–2750.
- Luxembourg, B., Delev, D., Geisen, C., Spannagl, M., Krause, M., Miesbach, W., Heller, C., Bergmann, F., Schmeink, U., Grossmann, R., Lindhoff-Last, E., Seifried, E., Oldenburg, J. & Pavlova, A. (2011) Molecular basis of antithrombin deficiency. *Thrombosis and Haemostasis*, **105**, 635–646.
- Luxembourg, B., D'Souza, M., Körber, S. & Seifried, E. (2015) Prediction of the pathogenicity of antithrombin sequence variations by in silico methods. *Thrombosis Research*, **135**, 404–409.
- Mahmoodi, B.K., Brouwer, J.L., Ten Kate, M.K., Lijfering, W.M., Veeger, N.J., Mulder, A.B., Kluin-Nelemans, H.C. & Van Der Meer, J. (2010) A prospective cohort study on the absolute risks of venous thromboembolism and predictive value of screening asymptomatic relatives of patients with hereditary deficiencies of protein S, protein C or antithrombin. *Journal of Thrombosis and Haemostasis*, **8**, 1193–1200.
- McCoy, A.J., Pei, X.Y., Skinner, R., Abrahams, J.P. & Carrell, R.W. (2003) Structure of beta-antithrombin and the effect of glycosylation on antithrombin's heparin affinity and activity. *Journal of Molecular Biology*, **326**, 823–833.
- Millar, D.S., Wacey, A.L., Ribando, J., Melissari, E., Laursen, B., Woods, P., Kakkar, V.V. & Cooper, D.N. (1994) Three novel missense mutations in the antithrombin III (AT3) gene causing recurrent venous thrombosis. *Human Genetics*, **94**, 509–512.
- Moore, G.W., de Jager, N. & Cutler, J.A. (2015) Development of a novel, rapid assay for detection of heparin-binding defect antithrombin deficiencies: the heparin-antithrombin binding (HAB) ratio. *Thrombosis Research*, **135**, 161–166.
- de la Morena-Barrio, M.E., Antón, A.I., Martínez-Martínez, I., Padilla, J., Miñano, A., Navarro-Fernández, J., Águila, S., López, M.F., Fontcuberta, J., Vicente, V. & Corral, J. (2012) Regulatory regions of SERPINC1 gene: identification of the first mutation associated with antithrombin deficiency. *Thrombosis and Haemostasis*, **107**, 430–437.
- de la Morena-Barrio, M.E., Buil, A., Antón, A.I., Martínez-Martínez, I., Miñano, A., Gutiérrez-Gallego, R., Navarro-Fernández, J., Aguila, S., Souto, J.C., Vicente, V., Soria, J.M. & Corral, J. (2013) Identification of antithrombin-modulating genes. Role of LARGE, a gene encoding a bifunctional glycosyltransferase, in the secretion of proteins? *PLoS ONE*, **8**, e64998.
- de la Morena-Barrio, M.E., Martínez-Martínez, I., de Cos, C., Wypasek, E., Roldán, V., Undas, A., vanScherpenzeel, M., Lefebvre, D.J., Toderici, M., Sevivas, T., España, F., Jaeken, J., Corral, J. & Vicente, V. (2016) Hypoglycosylation is a common finding in antithrombin deficiency in the absence of a SERPINC1 gene defect. *Journal of Thrombosis and Haemostasis*, **14**, 1549–1560.
- Mushunje, A., Zhou, A., Carrell, R.W. & Huntington, J.A. (2003) Heparin-induced substrate behavior of antithrombin Cambridge II. *Blood*, **102**, 4028–4034.
- Perry, D.J., Daly, M., Harper, P.L., Tait, R.C., Price, J., Walker, I.D. & Carrell, R.W. (1991) Antithrombin Cambridge II, 384 Ala to Ser. Further evidence of the role of the reactive centre loop in the inhibitory function of the serpins. *FEBS Letters*, **285**, 248–250.
- Reese, M.G., Eeckman, F.H., Kulp, D. & Haussler, D. (1997) Improved splice site detection in Genie. *Journal of Computational Biology*, **4**, 311–323.
- Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K. & Rehm, H.L.; ACMG Laboratory Quality Assurance Committee. (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine*, **17**, 405–424.
- Rossi, E., Za, T., Ciminello, A., Leone, G. & De Stefano, V. (2008) The risk of symptomatic pulmonary embolism due to proximal deep venous thrombosis differs in patients with different types of inherited thrombophilia. *Thrombosis and Haemostasis*, **99**, 1030–1034.
- Sakata, T., Okamoto, A., Mannami, T., Matsuo, H. & Miyata, T. (2004) Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese. *Journal of Thrombosis and Haemostasis*, **2**, 528–530.
- Schwarz, J.M., Cooper, D.N., Schuelke, M. & Seelow, D. (2014) MutationTaster2: mutation prediction for the deep-sequencing age. *Nature Methods*, **11**, 361–362.
- Tait, R.C., Walker, I.D., Perry, D.J., Islam, S.I., Daly, M.E., McCall, F., Conkie, J.A. & Carrell, R.W. (1994) Prevalence of antithrombin deficiency in the healthy population. *British Journal of Haematology*, **87**, 106–112.
- Teruel-Montoya, R., Rosendaal, F.R. & Martínez, C. (2015) MicroRNAs in hemostasis. *Journal of Thrombosis and Haemostasis*, **13**, 170–181.
- Tran, T.H., Bounameaux, H., Bondeli, C., Honkanen, H., Marbet, G.A. & Duckert, F. (1980) Purification and partial characterization of a hereditary abnormal antithrombin III fraction of a patient with recurrent thrombophlebitis. *Thrombosis and Haemostasis*, **44**, 87–91.
- Wells, P.S., Blajchman, M.A., Henderson, P., Wells, M.J., Demers, C., Bourque, R. & McAvoy, A. (1994) Prevalence of antithrombin deficiency in healthy blood donors: a cross-sectional study. *American Journal of Hematology*, **45**, 321–324.
- Wildeman, M., van Ophuizen, E., den Dunnen, J.T. & Taschner, P.E. (2008) Improving sequence variant descriptions in mutation databases and literature using the Mutalyzer sequence variation nomenclature checker. *Human Mutation*, **29**, 6–13.