Antithrombin is a member of the serine protease inhibitor (serpin) superfamily, which has a highly conserved structure, with three β-sheets (A–C) and eight to nine α-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).

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.
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 variants have been reported to be associated with antithrombin deficiency [http://www.hgmd.cf.ac.uk/ac; https://www1.imperial.ac.uk/departmentofmedicine/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 (≥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°C until use, and analysed after rapid thawing at 37°C. Genomic DNA was obtained from EDTA samples using the Qiacube® system (QIAGEN, Hilden, Germany).

Antithrombin assays

Antithrombin activity measurements were performed with an assay based on anti-FXa inhibition (INNOVANCE® 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® II & 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 promotor 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., 2010), 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 α-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 [Wilde 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.imperial.ac.uk/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, unraveling 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.

<table>
<thead>
<tr>
<th>Family</th>
<th>Sex</th>
<th>Age at VTE (years)</th>
<th>AT:act (%)</th>
<th>AT:ag ratio</th>
<th>Type</th>
<th>Location</th>
<th>Cytogenetic Nomenclature</th>
<th>Phenotypic Nomenclature</th>
<th>Reference</th>
<th>In silico prediction*</th>
<th>Tested family members</th>
<th>AT deficient/mutation</th>
<th>Normal/mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M</td>
<td>17</td>
<td>49</td>
<td>70</td>
<td>0-97</td>
<td>II PE</td>
<td>Exon 3 c.536T&gt;G</td>
<td>p.Phe179Cys</td>
<td>None</td>
<td>Pathogenic</td>
<td>1</td>
<td>1/1</td>
<td>0/0</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>24</td>
<td>56</td>
<td>67</td>
<td>0-93</td>
<td>I</td>
<td>Exon 5 c.857A&gt;C</td>
<td>p.Gln286Pro</td>
<td>None</td>
<td>Pathogenic</td>
<td>8</td>
<td>8/8</td>
<td>0/0</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>No VTE</td>
<td>72</td>
<td>96</td>
<td>0-77</td>
<td>II HBS**</td>
<td>Exon 2 c.218C&gt;T</td>
<td>p.Pro73Leu</td>
<td>Tran et al (1980)</td>
<td>Pathogenic</td>
<td>4</td>
<td>2/2</td>
<td>2/0</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>70</td>
<td>61</td>
<td>42</td>
<td>0-91</td>
<td>I</td>
<td>No mutation detected.</td>
<td></td>
<td>NA</td>
<td></td>
<td>2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>27</td>
<td>49</td>
<td>46</td>
<td>0-90</td>
<td>I</td>
<td>Exon 5 c.857A&gt;C</td>
<td>p.Gln286Pro</td>
<td>None</td>
<td>Pathogenic</td>
<td>4</td>
<td>3/3</td>
<td>1/0</td>
</tr>
<tr>
<td>H</td>
<td>M</td>
<td>17</td>
<td>47</td>
<td>41</td>
<td>0-88</td>
<td>I</td>
<td>Intron 4 c.763-1G&gt;A</td>
<td></td>
<td>None</td>
<td>Pathogenic</td>
<td>4</td>
<td>2/2</td>
<td>2/0</td>
</tr>
<tr>
<td>J</td>
<td>F</td>
<td>66</td>
<td>61</td>
<td>58</td>
<td>0-93</td>
<td>I</td>
<td>Exon 5 c.979dup</td>
<td>p.Val327Gly*16</td>
<td>None</td>
<td>Pathogenic†</td>
<td>5</td>
<td>2/2</td>
<td>3/0</td>
</tr>
<tr>
<td>K</td>
<td>F</td>
<td>No VTE</td>
<td>72</td>
<td>46</td>
<td>0-94</td>
<td>I</td>
<td>Exon 2 c.337_338delCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>M</td>
<td>29</td>
<td>68</td>
<td>99</td>
<td>0-83</td>
<td>II PE</td>
<td>Exon 4 c.749C&gt;T</td>
<td>p.Thr250Ile</td>
<td>None</td>
<td>Pathogenic</td>
<td>2</td>
<td>2/2</td>
<td>0/0</td>
</tr>
<tr>
<td>N</td>
<td>F</td>
<td>17</td>
<td>58</td>
<td>73</td>
<td>0-95</td>
<td>I</td>
<td>Exon 5 c.1033_1035del</td>
<td>p.Glu345del</td>
<td>Chowdhury et al (1993)</td>
<td>Pathogenic‡</td>
<td>2</td>
<td>2/2</td>
<td>0/0</td>
</tr>
<tr>
<td>O</td>
<td>F</td>
<td>21</td>
<td>51</td>
<td>103</td>
<td>1-01</td>
<td>II HBS**</td>
<td>Exon 7 c.1246G&gt;T</td>
<td>p.Ala416Ser</td>
<td>Perry et al (1991)</td>
<td>Pathogenic</td>
<td>2</td>
<td>1/1</td>
<td>1/0</td>
</tr>
<tr>
<td>P</td>
<td>F</td>
<td>26</td>
<td>69</td>
<td>48</td>
<td>0-96</td>
<td>I</td>
<td>No mutation detected.</td>
<td></td>
<td>NA</td>
<td></td>
<td>2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S</td>
<td>F</td>
<td>62</td>
<td>49</td>
<td>49</td>
<td>0-93</td>
<td>I</td>
<td>Exon 5 c.857A&gt;C</td>
<td>p.Gln286Pro</td>
<td>None</td>
<td>Pathogenic</td>
<td>6</td>
<td>3/3</td>
<td>3/0</td>
</tr>
<tr>
<td>U</td>
<td>M</td>
<td>No VTE</td>
<td>53</td>
<td>58</td>
<td>0-88</td>
<td>I</td>
<td>No mutation detected.</td>
<td></td>
<td>NA</td>
<td></td>
<td>2</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

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).
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 SERPIN1 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 SERPIN1. 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 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 I). 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 R 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 SERPIN1 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 SERPIN1 mutations have been described previously (Erdjument et al, 1988; Perry et al, 1991; Chowdhury et al, 1993).

In this study, almost half of the SERPIN1 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...
In conclusion, our study showed that, in most cases, antithrombin deficiency can be explained by an underlying SERPINC1 mutation, but that approximately 14% of clearly hereditary deficiencies cannot be explained. These results also highlight the importance of future research outside the conventional SERPINC1 gene approach. Finally, this study added several novel mutations to the already growing list of SERPINC1 mutations, thereby adding to our knowledge of the molecular background of antithrombin deficiency.

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

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