Antithrombin Dublin (p.Val30Glu): a relatively common variant with moderate thrombosis risk of causing transient antithrombin deficiency

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Summary
The key haemostatic role of antithrombin and the risk of thrombosis associated with its deficiency support that the low incidence of antithrombin deficiency among patients with thrombosis might be explained by underestimation of this disorder. It was our aim to identify mutations in SERPINC1 causing transient antithrombin deficiency. SERPINC1 was sequenced in 214 cases with a positive test for antithrombin deficiency, including 67 with no deficiency in the sample delivered to our laboratory. The p.Val30Glu mutation (Antithrombin Dublin) was identified in five out of these 67 cases, as well as in three out of 127 cases with other SERPINC1 mutations. Genotyping in 1593 patients with venous thrombosis and 2592 controls from two populations, revealed a low prevalent polymorphism (0.3 %) that moderately increased the risk of venous thrombosis (OR: 2.9; 95 % CI: 1.07–8.09; p= 0.03) and identified one homozygous patient with an early thrombotic event. Carriers had normal anti-FXa activity, and plasma antithrombin was not sensitive to heat stress or proteolytic cleavage. Analysis of one sample with transient deficit revealed a type I deficiency, without aberrant or increased latent forms. The recombinant variant, which lacked the two amino-terminal residues, had reduced secretion from HEK-EBNA cells, formed hyperstable disulphide-linked polymers, and had negligible activity. In conclusion, p.Val30Glu by affecting the cleavage of antithrombin’s signal peptide, results in a mature protein lacking the N-terminal dipeptide with no functional consequences in normal conditions, but that increases the sensitivity to be folded intracellularly into polymers, facilitating transient antithrombin deficiency and the subsequent risk of thrombosis.

Keywords
Antithrombin, deficiency, polymorphism, SERPINC1, thrombophilia

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Stroke, Systemic or Venous Thromboembolism

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Antithrombin is the primary haemostatic anticoagulant due both to the broad range of target procoagulant proteases and to its efficient mechanism of inhibition (1). Accordingly, complete deficiency caused embryonic lethality in mice (2), and resulted in spontaneous venous thrombosis in larvae of zebrafish (3). In humans, severe but also moderate antithrombin deficiency significantly increases the risk of thrombosis (4, 5). Actually, antithrombin deficiency was the first thrombophilic defect identified in 1965 by Olav Egeberg (6), and so far the strongest known thrombophilic factor (7). However, it is confusing why the incidence of congenital antithrombin deficiency is so low (below 1 %) in consecutive patients with venous thrombosis (8). Increasing evidence suggests that the incidence of antithrombin deficiency may be underestimated, particularly by the inability of current screening methods to detect pathological variants (9–13). Another mechanism that might increase the number of cases with thrombosis with underlying antithrombin defects might be the presence of a transient deficiency of this key anticoagulant. Actually, acquired antithrombin deficiency is also involved in thrombosis (14). We suggest that mutations affecting SERPINC1, the gene encoding antithrombin, might also cause transient antithrombin deficiency. Accordingly, we sequenced SERPINC1 in patients with a diagnosis of antithrombin deficiency even though a second analysis did not confirm the deficiency.
Methods

Patients and blood sampling

During the last 10 years, our centre recruited 214 blood samples of Caucasian subjects to characterise a potential antithrombin deficiency diagnosed by functional methods at the hospital of origin following the recommendations to avoid artefactual antithrombin deficiency such as the interference of pharmacological agents (especially heparin) and preanalytical variables, such as short-draw samples (11, 15, 16). Cases with liver disease or consumptive coagulopathy were also excluded (11). Blood was collected from the antecubital vein into citrate-tubes (109 mmol/l) and processed within 24 hours (h) after extraction. A new sample was obtained following the same requirements and immediately delivered to our centre and processed within 24–48 h after extraction. The first sample was obtained at least six months after the thrombotic event, when the patient was not under any anticoagulant therapy, except for P2, who was under oral anticoagulant therapy with acenocoumarol. No patient was evaluated under heparin treatment (first or subsequent samples).

Our study also included DNAs from 1520 patients with venous thrombosis and 2594 controls from two well defined case-control studies of different Caucasian populations, Spain and Denmark (17, 18).

All subjects included in this study gave their informed consent to enter the study, which was approved by the ethics committees for each participating institution and performed in accordance with the Declaration of Helsinki.

Molecular analysis of SERPINC1

Mutations in the SERPINC1, the gene encoding antithrombin, were determined by sequencing the seven exons and flanking regions, as well as the promoter region, using primers and conditions described elsewhere (19). Gross deletions affecting this gene were evaluated by multiplex ligation-dependent probe amplification (MLPA) using the P227-SerpinC1 probe mix and the Coffalyser software (MRC-Holland, Amsterdam, The Netherlands), following the conditions and analysis methods indicated by the manufacturer.

Genotyping of p.Val30Glu in the case-control studies was performed by fluorescence resonance energy transfer (FRET) in a Real-Time PCR System (LightCycler®480; Roche Molecular Biochemicals, Mannheim, Germany) using sequence specific probes (HHEX)CTGGGACTGCGTGACCTGTAC and [6FAM]CTGGGACTGCGGAGACCTGTAC (from Sigma-Aldrich Co, St. Louis, MO, USA). Validation of the genotype was done in all positive cases, including the homozygous patient, and in selected negative cases by direct sequencing.

Measurement of plasma antithrombin activity and antigen levels

Antithrombin activity (anti-FXa) was determined by chromogenic methods in citrated plasma (HaemosIL TH, Instrumentation Laboratory, Milan, Italy).

Antigen levels were measured by immunodiffusion or hom made ELISA.

Electrophoretic characterisation

Polyacrylamide gel electrophoresis (PAGE) in denaturing (under reducing and non-reducing conditions) and non-denaturing conditions (both in the presence and the absence of 6 M urea) was performed essentially as described elsewhere (20). After separation, proteins were transblotted onto a polyvinylidene difluoride membrane. Antithrombin was immunostained with rabbit anti-human antithrombin polyclonal antibody (Sigma-Aldrich), followed by donkey anti-rabbit IgG–horseradish peroxidase conjugate (GE Healthcare, Madrid, Spain), with detection via an ECL kit (Amersham Biosciences, Piscataway, NJ, USA).

Recombinant expression and purification of antithrombin variants

Recombinant antithrombin was constructed on the β-glycoform S137A antithrombin background in order to reduce glycosylation heterogeneity and to facilitate purification.

Site-directed mutagenesis of the pCEP4-S137A antithrombin plasmid, generously donated by Prof. J Huntington, was performed using the Stratagene Quick Change Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) as described previously (21) using the appropriate primers. Human Embryonic Kidney cells expressing the Epstein Barr Nuclear Antigen 1 (HEK-EBNA) were grown in DMEM with GlutaMAX-I medium (Invitrogen, Prat de Llobregat, Barcelona, Spain) supplemented with 5% fetal bovine serum (Sigma-Aldrich) to 60% confluence at 37 °C and 5% CO2 in a humidified incubator. We transfected 200 µg/ml of wild-type (WT) or mutant plasmids for 30 minutes (min) in OptiMEM with lipofectamine LTX (Invitrogen), following the manufacturer’s recommendations. After 24 h, cells were washed with PBS and exchanged into CD-CHO medium (Invitrogen) supplemented with 4 mM L-glutamine and 0.25 mg/ml Geneticin (Invitrogen). Cells were grown for 10 days and culture medium was collected every two days for purification.

Purification of recombinant proteins secreted to the conditioned medium was performed by heparin chromatography followed by ion-exchange chromatography, as described elsewhere (21). Cells were extensively washed with sterile PBS and then lysed with 50 µl of lysis buffer (10 mM TrisHCl, 0.5 mM DTT, 0.035% SDS, 1 mM EGTA, 50 mM sodium fluoride, 50 µM sodium orthovanadate, 5 mM benzamidine and 20 mM phenylmethylsulphonyl fluoride). The lysate was evaluated by SDS-PAGE and Western blot, as indicated before.

Proteomic analysis

The molecular weight was determined by HPLC-ESI-MS TOF (HPLC Agilent 1100 Series; Agilent 6100 Series TOF Mass Spectrometer; Agilent Technologies) using an electrospray interface. Recombinant native and elastase-cleaved WT and p.Val30Glu
variant were reduced with 50 µM DTT, and then injected onto a Supelco Discovery BioWide C5 HPLC column (5 µm, 100 × 2.1 mm, Sigma-Aldrich). External calibration of the spectrometer was performed using the monoisotopic peak of two standard peptides (bradykinin, 757.3997 m/z, and ACTH, 2465.1989 m/z) from the ProteoMass™ Peptide MALDI-MS Calibration Kit (Sigma-Aldrich). Two proteins were used as control (Cytochrome C and Carbonic anhydrase, Sigma-Aldrich). Data were recorded and processed with Agilent MassHunter Workstation Qualitative Analysis Software (B.03.01). The intact molecular weight of each protein was calculated from the main mass spectrum using the deconvolution algorithm.

Calorimetry
The heat capacity (Cp) of samples was recorded over a temperature range of 10–130°C, by using differential scanning calorimetry (VP-DSC, MicroCal, LLC, Northampton, MA, USA), employing two fixed cells, a reference cell, and a sample cell, essentially as described elsewhere (22). The measurements were carried out with a microcalorimeter at the scan rate of 60°C/h using recombinant WT and p.Val30Glu antithrombins. Data were analysed using the ORIGIN DSC software that was provided by MicroCal Inc (Northampton, MA, USA).

Edman sequencing
Edman sequencing of the mutants purified from recombinant variant were performed under reducing conditions with Applied Biosystems Procise 494 equipment (Foster City, CA, USA).

Protease treatment
One µl of plasma from carriers of SERPINC1 mutations and controls was treated with 0.25 U of thrombin (Calbiochem, San Diego, CA, USA) for 15 min at 37 or 42°C in presence of unfractionated heparin (5 U, Rovi SA, Barcelona, Spain). The effect of this treatment on the generation of thrombin-antithrombin complexes and cleaved antithrombin was evaluated by immunoblot analysis after SDS-PAGE under non-reducing conditions following the procedures described before. Similar treatment with 5 U of neutrophil elastase (Calbiochem) and unfractionated heparin (5 U) was also evaluated. The effect of this treatment on the generation of cleaved antithrombin was evaluated by immunoblot analysis after non-denaturing-urea-PAGE following the procedures described before.

Statistical analysis
The strength of the association of genetic defects with the occurrence of disease was estimated using 2 × 2 tables and calculating the odds ratio (OR) with the Epilnfo software (Centre for Disease Control, Atlanta, GA, USA) and the Cornfield method for the calculation of 95% confidence intervals (CI). Logistic regression models adjusted for individual risk factors (age, gender, factor V Leiden and prothrombin G20210A) were calculated using Statistical Package for the Social Sciences (SPSS), version 15.0, to predict the risk of thrombosis of the SERPINC1 p.Val30Glu mutation.

Results
Identification of the SERPINC1 p.Val30Glu mutation
Two hundred fourteen patients were recruited in the haematology unit of 30 hospitals based on functional anti-FXa activity below the reference value (80%). The analysis of anti-FXa activity in a new sample performed in our laboratory failed to confirm the suspected antithrombin deficiency in 67 cases.

Molecular analysis of SERPINC1 (exons and flanking regions and gross gene defects) was done in these 214 patients. Interestingly, eight patients carried the p.Val30Glu (nomenclature according to the Human Genome Variation Society HGVS; p.Val30Glu in the mature protein), responsible for the antithrombin

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Thrombosis (Age)</th>
<th>Familial history of thrombosis</th>
<th>Other risk factors*</th>
<th>Anti-FXa activity</th>
<th>SERPINC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>F</td>
<td>36</td>
<td>PE (27)</td>
<td>Yes</td>
<td>No</td>
<td>50/52</td>
<td>p.Val30Glu &amp; p.Arg425Cys</td>
</tr>
<tr>
<td>P2</td>
<td>M</td>
<td>73</td>
<td>PE (30) R</td>
<td>Yes</td>
<td>No</td>
<td>51/50</td>
<td>p.Val30Glu &amp; p.Leu317Phe</td>
</tr>
<tr>
<td>P3+</td>
<td>F</td>
<td>34</td>
<td>Stroke (30)</td>
<td>No</td>
<td>No</td>
<td>60/58</td>
<td>p.Val30Glu &amp; Deletion exon 7</td>
</tr>
<tr>
<td>P4</td>
<td>M</td>
<td>38</td>
<td>PE (37)</td>
<td>No</td>
<td>No</td>
<td>73/89</td>
<td>p.Val30Glu</td>
</tr>
<tr>
<td>P5</td>
<td>M</td>
<td>34</td>
<td>DVT (33)</td>
<td>Yes</td>
<td>No</td>
<td>70/100/70</td>
<td>p.Val30Glu</td>
</tr>
<tr>
<td>P6</td>
<td>M</td>
<td>63</td>
<td>DVT (61)</td>
<td>No</td>
<td>No</td>
<td>57/75</td>
<td>p.Val30Glu</td>
</tr>
<tr>
<td>P7</td>
<td>F</td>
<td>25</td>
<td>Stroke (24)</td>
<td>Yes</td>
<td>No</td>
<td>68/84</td>
<td>p.Val30Glu</td>
</tr>
<tr>
<td>P8</td>
<td>F</td>
<td>26</td>
<td>RVT (20) R</td>
<td>Yes</td>
<td>No</td>
<td>60/102</td>
<td>p.Val30Glu</td>
</tr>
</tbody>
</table>

* Factor V Leiden (FVL), Prothrombin G20210A (PT), protein C or S deficiency, antiphospholipid antibodies. † This patient also has Down syndrome. PE: Pulmonary embolism; DVT: Deep venous thrombosis; RVT: Retinal vein thrombosis. R: Recurrence.
Dublin variant (23). The p.Val30Glu mutation was present in three out of 127 patients with one additional SERPINC1 gene defect. All these cases had manifest antithrombin deficiency in all tested samples (50–60%) (Table 1). The first case (P1) was a 36-year-old female who developed a pulmonary embolism when she was 27 years old. The patient also carried a p.Arg425Cys mutation responsible for Antithrombin Northwick Park, a type IIa variant (24). The second case (P2), a 73-year-old male with type I deficiency, developed early and recurrent thrombosis, including a pulmonary embolism. P2 also carried the p.Leu317Phe change, a new mutation that affects a highly conserved residue located at s3B. The same residue has been found mutated to Ser in a patient with a type I deficiency (25). A cousin of P2 also carried the same combination of SERPINC1 mutations (p.Val30Glu and p.Leu317Phe) and he developed early and recurrent thrombosis. The third patient (P3) was a 34-year-old woman who developed an ischaemic stroke when she was 30 years old. This patient had a type I antithrombin deficiency and also carried a deletion of exon 7, a genetic defect previously described in a patient with type I deficiency (26). Thrombosis in all these patients with two SERPINC1 mutations was idiopathic.

The other five patients only carrying the p.Val30Glu mutation (P4-P8), all heterozygous, had a particular feature: they had mild to moderate antithrombin deficiency in the sample evaluated at the original hospital, but in all cases the sample delivered to our laboratory had nearly normal values according to our anti-FXa chromogenic procedure (90.0 ± 11.3%) (Table 1). Thus, the p.Val30Glu mutation was present in 7.5% of cases in which the suspected antithrombin deficiency was not confirmed in the sample evaluated in our laboratory. Further demographic, analytical and clinical features of these five patients are also shown in Table 1.

**Case control studies**

The identification of a high number of carriers of the p.Val30Glu mutation among patients with thrombosis and at least one positive result in tests for antithrombin deficiency, and the suspected transient antithrombin deficiency observed in carriers, encouraged to determine the thrombotic consequences of the p.Val30Glu mutation in an epidemiologic study. Thus, this variation was genotyped in 1593 patients with venous thrombosis and 2592 controls from two different populations, Spain and Denmark (Suppl. Table 1, available online at www.thrombosis-online.com).

The p.Val30Glu was a low prevalent variation in both populations (Table 2), with a minor allele frequency, 0.001, similar to that described in the European population for this polymorphism (rs2227624) (0.002) (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2227624). Interestingly, in both populations the prevalence of the p.Val30Glu variation was higher in patients with venous thrombosis, and globally, this polymorphism mildly but significantly increased the risk of venous thrombosis (OR: 2.69; 95%CI: 1.04–6.96; p= 0.020) (Table 2). The association was maintained after adjusting by age, sex and prothrombotic polymorphisms (Factor V Leiden and prothrombin G20210A) (OR: 2.94; 95%CI: 1.07–8.09; p= 0.037) (Table 2). Interestingly, one Spanish 32-year-old male patient, without additional thrombophilic factors, who developed an idiopathic deep venous thrombosis when he was 28 years old, carried this variation in homozygous state. No carrier of the p.Val30Glu mutation was diagnosed of antithrombin deficiency, as all had anti-FXa values within the normal range (80–120%), also the case with the mutation in homozygous state, a patient that had 92% of anti-FXa activity.

**Analysis of plasma antithrombin in carriers of the SERPINC1 p.Val30Glu mutation**

Electrophoretic analysis of plasma antithrombin in the sample of p.Val30Glu carriers delivered to our laboratory did not reveal significant differences compared with non-carrier healthy controls. Thus, levels of plasma antithrombin were similar in carriers and non-carriers, and neither dimers, nor polymers, nor aberrant forms were detected by non-denaturing PAGE analysis in carriers of this mutation. Moreover, the levels of the latent form of antithrombin in carriers of this variation, identified by non-denaturing-PAGE in presence of urea, were in the normal range and did not differ from that observed in non-carriers healthy carriers.

**Table 2: Prevalence of p.Val30Glu and risk of thrombosis associated to this variation in the case-control studies.**

<table>
<thead>
<tr>
<th></th>
<th>Spain Cases (N=540)</th>
<th>Spain Controls (N=540)</th>
<th>Denmark Cases (N=1615)</th>
<th>Denmark Controls (N=1615)</th>
<th>All Cases (N=1520)</th>
<th>All Controls (N=2594)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val/Val</td>
<td>972 (99.2%)</td>
<td>975 (99.6%)</td>
<td>537 (99.4%)</td>
<td>1612 (99.8%)</td>
<td>1509 (99.2%)</td>
<td>2587 (99.7%)</td>
</tr>
<tr>
<td>Val/Glu</td>
<td>7 (0.7%)</td>
<td>4 (0.4%)</td>
<td>3 (0.6%)</td>
<td>3 (0.2%)</td>
<td>10 (0.7%)</td>
<td>7 (0.3%)</td>
</tr>
<tr>
<td>Glu/Glu</td>
<td>1 (0.1%)</td>
<td>0</td>
<td>0</td>
<td>1 (0.1%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Crude OR</td>
<td>2.01 (0.62–6.68)</td>
<td>0.133</td>
<td>3.00 (0.60–14.92)</td>
<td>0.104</td>
<td>3.00</td>
<td>0.020</td>
</tr>
<tr>
<td>Adjusted OR*</td>
<td>2.67 (0.70–10.21)</td>
<td>0.153</td>
<td>2.71 (0.52–14.03)</td>
<td>0.235</td>
<td>2.94</td>
<td>0.037</td>
</tr>
</tbody>
</table>

*Adjusted by sex, age and prothrombotic polymorphisms (Factor V Leiden and prothrombin G20210A).*
controls (Suppl. Figure 1, available online at www.thrombosis-online.com).

Particular interest had patient P5, as we recruited a new sample with reduced anti-FXa values (70%). Electrophoretic analysis revealed reduced antigen levels, without aberrant forms or increased levels of latent antithrombin in the sample with reduced anti-FXa activity compared with that displaying normal anti-FXa activity (Figure 1).

A potential increased conformational instability of plasma antithrombin in carriers of the p.Val30Glu mutation was analysed by incubating plasma at 42°C for up to 72 h and evaluating residual anti-FXa activity and the proportion of the latent conformation by non-denaturing PAGE with urea and Western blot. As shown in Suppl. Figure 2 (available online at www.thrombosis-online.com), variations of these parameters were comparable in carriers and non-carriers of the p.Val30Glu mutation.

We also evaluated a possible substrate behaviour of this variant to thrombin by incubating plasma of carriers and controls with thrombin in the presence of unfractionated heparin, as indicated in the material and methods section. Evaluation of thrombin-antithrombin complexes and cleaved antithrombin by SDS-PAGE under non-reducing conditions showed similar results in carriers and non-carriers (Suppl. Figure 3, available online at www.thrombosis-online.com). Finally, a potentially increased sensitivity to protease attack was discarded by treatment of plasma with neutrophil elastase and unfractionated heparin as the levels of cleaved antithrombin did not significantly increase in carriers of the p.Val30Glu mutation (Suppl. Figure 4, available online at www.thrombosis-online.com).

Recombinant expression of p.Val30Glu

In order to explain the apparent normal activity, levels and stability of the plasma variant but the potential transient antithrombin deficiency and the high risk of thrombosis associated to this mutation, we evaluated the consequences of the p.Val30Glu mutation in a recombinant model. The p.Val30Glu mutation impaired five-fold the secretion of the variant from HEK-EBNA cells to the conditioned medium (Figure 2). Although the variant showed similar electrophoretic mobility than WT antithrombin in SDS-PAGE under reducing conditions (Figure 2), it had negligible inhibitory activity. Thus, conditioned medium of p.Val30Glu had no anti-FXa activity when evaluated by using chromogenic methods (data not shown) and only slight traces of thrombin-antithrombin complexes were observed in SDS-PAGE and Western blot after incubation with thrombin and unfractionated heparin when using high proportions of conditioned medium of cells transfected with the mutant plasmid (Figure 3).

In order to explain the absence of inhibitory activity for the p.Val30Glu variant produced in HEK-EBNA cells, we first evaluated the consequences of this mutation in the proteolytic cleavage of the signal peptide in these cells. N-terminal sequencing of antithrombin purified from conditioned medium revealed a single sequence: S-P-V-D-I, identical to that described in the Dublin variant purified from the plasma of carriers (27), thus lacking the N-terminal dipeptide, His-Gly. Interestingly, calorimetric analysis of WT and p.Val30Glu recombinant molecules revealed that the variant had a hyperstable conformation (Figure 4). Validating this result, SDS-PAGE under non-reducing conditions revealed high proportions of disulphide-linked polymers for the p.Val30Glu recombinant variant from the conditioned medium (Figure 3A and Figure 5A). The intracellular variant also had the same pattern of polymers (Figure 5B). The variant also had a monomer...
with electrophoretic mobility similar to that of cleaved antithrombin (▶Figure 3 and ▶Figure 5). However, Edman's analysis revealed no further N-terminal sequences and, proteomic analysis under reducing conditions of the variant revealed no significant change on the molecular weight compared with the recombinant WT molecule (55762 Da and 55678 Da, respectively) while the treatment of recombinant WT antithrombin with elastase rendered a smaller molecule (54720 Da). Thus, this could be the electrophoretic mobility of the recombinant monomer variant lacking the N-terminal dipeptide, although a potential latent conformation of this monomer can not be discarded.

Discussion

The search for congenital thrombophilic factors has identified few new defects despite hundreds of studies involving thrombophilic families and thousands of patients with venous thrombosis (7). One potential explanation of these frustrating results could be that all strategies have been focused to find mutations leading to a permanent functional defect. The most recently described thrombophilic disorders have these features (28, 29). We propose the existence of congenital risk factors of thrombosis able to have functional consequences that occur only under specific conditions. Our hypothesis suggests that these mutations may cause a transient prothrombotic state. Unfortunately, these defects may only be detected if the sample to be analysed is collected under the conditions triggering the deleterious consequences of the mutation or by molecular studies. The key role of antithrombin in the haemostatic system and the prothrombotic relevance of its deficiency encouraged us to test our hypothesis among patients with a suspicion of antithrombin deficiency, particularly if the second test failed to verify the deficiency. Thus, we sequenced the SERPINC1 in 67 patients with one positive determination of antithrombin deficiency using functional methods that, however, had normal anti-FXa activity in the sample evaluated in our laboratory. Multiple mechanisms might explain these contradictory results: differences in reagents or procedures used to diagnose the deficiency by the two laboratories involved, pharmacological agents

![Figure 3: Anticoagulant activity of recombinant antithrombins. A) SDS-PAGE under non-reducing conditions and immunoblot of conditioned medium or cell lysates of HEK-EBNA cells transfected with the pCEP4-S137A antithrombin plasmid (WT) or the same plasmid with the p.Val30Glu mutation (V30E) in presence and absence of thrombin and unfractionated heparin. B) SDS-PAGE under reducing conditions and immunoblot of conditioned medium, in presence of thrombin and unfractionated heparin. For a better comparison, five-fold conditioned medium of cell transfected with the p.Val30Glu mutation were loaded. Thrombin-Antithrombin complexes (T-AT) native antithrombin (N-AT) and cleaved antithrombin (C-AT) are pointed by arrows.](image)

![Figure 4: Calorimetry analysis of recombinant p.Val30Glu (V30E) and wild-type (WT) antithrombins.](image)
Figure 5: Identification of disulphide-linked polymers caused by the p.Val30Glu mutation in the recombinant model. A) Conditioned medium of HEK-EBNA cells transfected with the pCEP4-S137A antithrombin plasmid (WT) or the same plasmid with the p.Val30Glu mutation (V30E) and grown at 37°C for 48 h. For a better comparison, five-fold conditioned medium of cell transfected with the p.Val30Glu mutation were loaded. B) Intracellular antithrombin of the same samples. Antithrombin was detected by SDS-PAGE under not reducing conditions and Western blot.

(especially heparin), preanalytical variables, such as short-draw samples, errors of identification of samples, acquired deficiency, but also our hypothesis, a mutation that facilitates a transient deficiency. Interestingly, a recurrent mutation, p.Val30Glu, responsible for the antithrombin Dublin variant was present in 7.5% of these patients.

Although the p.Val30Glu mutation was identified in a family with venous thrombosis 27 years ago (23), the clinical relevance of this genetic variation remains unclear. Thus, although it is considered as a polymorphism (rs2227624), few reports have identified this mutation among patients with venous thrombosis (31, 32). Interestingly, a recent meta-analysis of available genome-wide association studies (GWAS) performed in venous thrombosis showed that this polymorphism significantly increases (2.3 fold) the risk of thrombosis, almost the same result obtained in our case-control study (32). However, an excellent study purified and characterised the variant from the plasma of carriers, demonstrating that antigen levels, heparin affinity and anticoagulant activity of the variant were not affected by this mutation although it redirects signal peptidase cleavage to a site two amino acids downstream into the mature protein as it lacks the N-terminal dipeptide, His-Gly (27).

Interestingly, in all five patients from our study in which this mutation was the only molecular defect in SERPINC1, the second sample evaluated in our laboratory had anticoagulant activity within the normal range. Moreover, all carriers identified in the case control studies, including the homozygous one, had normal anti-FXa values. In other words, this mutation seems to cause a transient antithrombin deficiency. This result may explain why this mutation is not frequently identified in studies selecting patients with permanent antithrombin deficiency (33, 34). However, according to the results obtained in our case-control study, the significant risk of thrombosis associated with this mutation supports that the p.Val30Glu mutation should have a deleterious effect. We aimed to identify the prothrombotic mechanism associated with this mutation, as it could also explain why the antithrombin activity is only impaired at certain time points.

Whereas the analysis of plasma antithrombin in carriers suggests that the variant in plasma has no increased sensitivity to heat stress or proteolytic attack, which do not clarify the prothrombotic mechanism of this mutation, the study of the recombinant variant (which also lacks the N-terminal dipeptide demonstrating that the p.Val30Glu mutation redirects signal peptidase cleavage to a site two amino acids downstream into the mature protein in human cells) gave key clues about the potential mechanisms leading to a temporary inactivation of this variant. Antithrombin, as a member of the serpin superfamily, is folded into a metastable native conformation, which is required for its effective inhibitory mechanism but that also makes antithrombin vulnerable to even missense mutations, particularly those affecting mobile regions of the molecule. The mutation of these residues favors an aberrant folding of serpins with pathological consequences (35). Moreover, native antithrombin, with a reactive centre loop (RCL) partially inserted, spontaneously transforms into a hyperstable non-inhibitory latent conformation with the RCL forming the central strand in the A sheet. Indeed, up to 5% of plasma antithrombin has latent conformation (36), and higher temperature or other factors, increase the proportion of this non-inhibitory conformation (37). Mutations like the p.Pro112Ser or p.Gly456Arg cause the folding of the variant antithrombin into disulphide-linked polymers, which are retained intracellularly (20). The effect of these mutations is permanent and carriers always had antithrombin deficiency. We propose that the p.Val30Glu mutation does not cause but facilitates the folding into hyperstable conformations, mainly disulphide-linked polymers. This transient conformational consequence has been described previously for two SERPINC1 mutations, p.Thr117Met (antithrombin Wibble) and p.Asp219Asn (antithrombin Rouen VI), which facilitate the transition to the latent form on incubation with moderately increased temperatures (42°C) (38, 39). Thus, antithrombin deficiency in carriers was only detected under conformational stress conditions, such as increased body temperatures of fever, which have been described underlying the thrombotic event in some carriers (39). The p.Val30Glu mutation has the same final functional effect and deleterious consequences as that described for the Wibble and Rouen VI variants: nearly normal antigen levels and function under normal conditions, but increased transition to the non-inhibitory conformations under certain conditions. However, two differences should be pointed out: i) the Dublin variant is relatively prevalent in the general population, ii) the p.Val30Glu mutation only caused instability during the folding process, as if the variant is correctly folded to the native conformation, the absence of the N-terminal
Congenital thrombophilic factors cause a permanent hypercoagulable state detected by functional methods included in thrombophilic tests. Antithrombin deficiency is the strongest known thrombophilic factor, but its incidence may be underestimated, particularly by the inability of current screening methods to detect pathological variants.

What is known about this topic?
- Congenital thrombophilic factors cause a permanent hypercoagulable state detected by functional methods included in thrombophilic tests.
- Antithrombin deficiency is the strongest known thrombophilic factor, but its incidence may be underestimated, particularly by the inability of current screening methods to detect pathological variants.

What does this paper add?
- The SERPINC1 p.Val30Glu mutation is frequent (7.5%) among cases with conflictive antithrombin deficiency, with positive and negative results in different samples.
- The SERPINC1 p.Val30Glu mutation is relatively frequent in the general population (0.3%) and mildly increases the risk of venous thrombosis (OR: 2.7).
- This mutation, by modifying the cleavage of the signal peptide, renders a variant lacking the N-terminal His-Gly dipeptide which folds into a native conformation with thermal stability and function similar to the wild-type antithrombin. However, this mutation also facilitates its intracellular transition to hyperstable not functional conformations.
- Our study introduces the term congenital transient thrombophilia; prothrombotic mutations with deleterious consequences only under specific conditions, which make them hardly detectable by using current functional thrombophilic tests.
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Author contributions
Contribution: JNF and JC designed the research; TSS, CC, NFM, PL, PM, KO, SRK, and JCS provided study materials or patients and collected clinical information; JP and MEM generated recombinant antithrombins; JNF, JC and VV wrote the paper. All authors provided final approval of the manuscript.

Conflicts of interest
None declared.

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