Congenital antithrombin deficiency in patients with splanchnic vein thrombosis

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Abstract

Background and aims: Splanchnic vein thromboses (SVT) are a rare condition that can be life-threatening. The most severe thrombophilia associated to SVT is antithrombin (AT) deficiency, usually caused by SERPINC1 mutations. Although transitory AT deficiencies and congenital disorders of the N-glycosylation pathways (CDG) have been recently reported as causes of AT deficiency, the current AT clinical screening still only includes anti-FXa activity. This study aims to (a) improve the detection of AT deficiency in SVT and (b) characterize the features of AT deficiency associated with SVT.

Methods: The study was performed in 2 cohorts: (a) 89 SVT patients with different underlying etiologies but in whom AT deficiency had been ruled out by classical diagnostic methods; and (b) 271 unrelated patients with confirmed AT deficiency and venous thrombosis. AT was evaluated by functional (anti-FXa and anti-FIIa) and immunological methods (ELISA, crossed immunoelectrophoresis, western blot), and SERPINC1 sequencing was performed.

Results: In 4/89 patients (4.5%) additional alterations in AT were found (two had SERPINC1 mutations, one had a specific variant causing transient AT deficiency and one patient had CDG). In 11 of the 271 patients (4.1%) with AT deficiency and thrombosis, thrombosis was located at the splanchnic venous territory.

Conclusions: Antithrombin deficiency may be underdiagnosed by current clinical screening techniques. Therefore, a comprehensive AT evaluation should be considered in cases of rethrombosis or doubtful interpretation of anti-FXa activity levels. SVT is a relatively common localization of the thrombotic event in patients with congenital AT deficiency.

KEYWORDS
antithrombin, splanchnic thrombosis, venous thrombosis

Abbreviations: AT, antithrombin; BCS, Budd Chiari syndrome; CDG, congenital disorders of glycosylation; FIIa, active factor II; Fxa, active factor X; MAF, minor allele frequency; PVT, portal vein thrombosis; SVT, splanchnic vein thromboses.

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INTRODUCTION

Thrombosis of the splanchnic veins (SVT) is a rare condition with a prevalence of less than 5 cases every 10,000 inhabitants. However, despite being rare, SVT are a complex condition that can be life-threatening and lead to severe complications such as portal hypertension or intestinal ischemia.1

The most frequent causes of SVT are major thrombophilic disorders and local factors, the latter being mainly abdominal inflammatory processes such as pancreatitis and surgical interventions. It is remarkable that in up to 30% of patients with a local factor there is also an underlying thrombophilic disorder, acting the local inflammation as trigger for the development of thrombosis.2

Regardless availability of better diagnostic tools such as detection of Calretulin or JAK2 mutations,3,4 in around 30% of cases it is not possible to identify the thrombosis’ etiology and patients are finally classified as having an idiopathic thrombosis. Correctly classifying SVT thrombosis according to its etiology (major thrombophilic disorders vs local inflammation/idiopathic thrombosis) is highly relevant. Indeed, current guidelines only recommend long-term anticoagulation to prevent re-thrombosis in those patients with underlying thrombophilic disorders. On the contrary, patients with idiopathic thrombosis or those associated only to local factors will only receive anticoagulation in the setting of acute thrombosis.5,6 Interestingly, however, despite following these current guidelines, recent evidence shows a relevant incidence of new thrombotic events in patients with idiopathic thrombosis not treated with anticoagulants.7

Antithrombin (AT) deficiency is one of the most severe hereditary thrombophilia.8-10 Homozygote AT mutations are lethal disorders that cause intrauterine foetal death while heterozygote mutations lead to a hypercoagulable state with high probability of developing thrombosis.11

Antithrombin deficiencies have been classified into two types: in type I, levels of AT are low; in type II, AT levels are within the normal range but the AT molecule has impaired or null anticoagulant activity.12 Several AT deficiency mechanisms have been described, being the most frequent genetic variants in SERPINC1 (the gene encoding antithrombin)13 that account for up to 80% of the cases of AT deficiencies.

The real incidence of SVT among carriers of AT deficiency has not been previously established.14 However, from a clinical point of view, it is striking that despite the high prothrombotic risk of AT deficiency, the reported incidence of this alteration is very low among patients with splanchnic venous thrombosis, thus suggesting that AT deficiencies might be underestimated. There are increasing evidences on the low sensitivity of the current functional diagnostic tests to detect pathogenic mutations causing AT deficiency.15 Moreover, it has been reported that the pathogenic effect of some SERPINC1 mutations might be exacerbated by environmental factors, leading to transient AT deficiency that would only be detected by functional methods. This would be the case of SERPINC1 mutation p.Val30Glu.15 Patients with this mutation would have normal levels and function of AT in baseline conditions but with a higher conformational susceptibility to stress, which would lead to deficiency of AT.15 Similarly, post-translational defects such as alterations and reduction of the N-glycosylation pathways16-18 caused by congenital disorders of glycosylation (CDG), which might be not detected by conventional functional methods, are also known to impair AT function.17

Rendering the interpretation of AT levels even more difficult, acquired causes of low AT are also a common cause of low AT levels, but importantly do not lead to hypercoagulable states. Therefore, when finding low levels of AT (<80%) it is essential to exclude potential acquired etiologies before diagnosing hereditary AT deficiency. Several conditions can lead to low levels of AT and must be taken into account in the differential diagnosis (ie liver disease can decrease AT levels because of decreased hepatic synthesis; recent or active thrombosis lead to a general decrease in coagulation factors).

If AT deficiency is undetected and misdiagnosed, patients requiring anticoagulation could remain untreated with the consequent risk of re-thrombosis, not only SVT but also in other vascular territories. Diagnosing these patients would allow a correct risk stratification which could change the decision of prescribing chronic anticoagulation.

In this framework, we have designed the current study with 2 objectives: (a) to evaluate if it is possible to improve the detection of AT deficiency in patients with SVT using new diagnostic techniques (b) to report the features of AT deficiency that associate with SVT.

MATERIALS AND METHODS

The study was performed in accordance with the International Guideline for Ethical Review of Epidemiological Studies and principles of the Declaration of Helsinki and approved by the ethics committee of Hospital Clinic de Barcelona and Hospital Universitario Reina Sofía de Murcia. Written informed consent from all patients was provided.
This study is based in two different cohorts:

Cohort 1. Since 2003, all consecutive patients with SVT seen at Hospital Clinic de Barcelona (n = 284) were asked for permission to obtain a blood sample for research purposes. Blood samples were stored at IDIBAPS Biobank facilities. As shown in Figure S1, of these 284 patients 5 were diagnosed with AT deficiency (1.8%). Among the remaining 279 patients, we randomly selected 90 patients trying to represent the 3 main underlying etiologies (thrombophilia, local factor and idiopathic). Finally, one patient was discarded because of inadequately stored biobank sample. Thus, 89 patients with SVT (68 with portal vein thrombosis [PVT] and 21 with Budd Chiari syndrome [BCS]) who had a complete etiological diagnostic work-up ruling out AT deficiency and from whom we had samples in the Biobank, were included in the study. AT deficiency had been clinically evaluated by a functional chromogenic method (anti-active factor X (FXa) activity test). Concomitant testing of other hepatic factors such as protein C and protein S had also been performed to assess acquired low AT levels. For each subject, clinical, haematological and laboratory data were collected. As detailed below, AT activity was assessed again at the biobank samples evaluating both anti-FXa and anti-active factor II (FIIa) activity. Also, total DNA was extracted from biobank peripheral blood samples by salting out procedures. In those patients showing AT levels below 80% (either by anti-FXa and/or by anti-FIIa activity), a genetic study of SERPINC1 was performed (detailed below). Moreover, in all patients of this cohort analysis of plasma AT by western blot aiming to find aberrant forms of AT including hypoglycosylation and search of mutations responsible for transient AT deficiency was evaluated as described below.

Cohort 2. Three hundred forty-seven patients with previous thrombotic events (in any territory) and suspected AT deficiency based on the results of thrombophilic tests were referred during 22 years (1996-2018) to Hospital Universitario Reina Sofía de Murcia, the Spanish reference centre for this disorder. Plasma and DNA were collected and/or extracted as stated above and properly stored. Biochemical and functional characterization of plasma AT as well as genetic analysis were performed as detailed below. This study was performed in 271 patients with confirmed AT deficiency among the 347 referred patients. Familial studies were done in 206 cases.

2.2 | Genetic analysis

Genetic SERPINC1 variants were identified by sequencing the 7 exons and flanking regions, or the whole gene by Sanger’s or NGS methods as described before. In Cohort 1, genetic analysis was performed in every patient with AT anti-FXa or anti-FIIa activity below 80%: 21 SVT patients with two evidences of antithrombin deficiency independently of reduced levels of other hepatic proteins (all sequenced by Sanger’s method). Moreover, exons 2 and 7 were sequenced in all 89 patients with SVT by using Sanger sequencing. In Cohort 2, all 347 patients with congenital antithrombin deficiency underwent SERPINC1 sequencing (283 sequenced by Sanger’s method and 64 by NGS). Positive findings by NGS were always validated by Sanger sequencing.

Gross rearrangements were assessed by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA Kit P227 SerpinC1 (MRC-Holland). Mutations were described following the Human Genome Variation Society Guidelines (http://varnomen. hgv s.org/recommendations/). The GenBank NM_000488.3 cDNA sequence was used as reference. Where available, HGMD (Human Gene Mutation Database) accession numbers are mentioned.

2.3 | Biochemical and functional characterization of plasma antithrombin

Antithrombin anti-FXa and anti-FIIa activity were determined in citrated plasma by chromogenic methods. Values were expressed as percentages referred to a pool of 100 healthy subjects. AT deficiency was considered when values were below 80%. Antigen levels were measured by rocket immunoelectrophoresis and/or ELISA (enzyme-linked immunosorbent assay). These methods were described in previous manuscripts.

Analysis of plasma AT forms included crossed immunoelectrophoresis and polyacrylamide gel electrophoresis under different conditions (denaturing and not denaturing) followed by immunological detection using conditions and reagents previously described.

Hypoglycosylation of AT and other hepatic proteins (α1-antitrypsin and transferrin) in plasma was evaluated by western blot or HPLC (high-performance liquid chromatography) as described elsewhere.

The reported results were performed in samples collected long after the acute event in the cohort of patients with congenital AT deficiency (mean 42 ± 76 months).

2.4 | Alignment of antithrombin protein sequences among species

The alignment of antithrombin protein sequences with up to 49 species was performed by uniprot tool (https://www.uniprot.org/align/) and visualized by Jalview version 2.

2.5 | Statistical analysis

Quantitative data are expressed as median and range. Qualitative data are expressed as percentages.

3 | RESULTS

Cohort 1 Report of antithrombin deficiency in a large cohort of patients with SVT (PVT and BCS) in which this disorder had been originally ruled out.
Table 1 shows baseline characteristics of Cohort 1, including the etiology and the different thrombophilia present in this cohort. Overall, the 3 main etiologies were similarly represented: 28 patients (31.5%) had a known thrombophilia, 26 (29.2%) were associated to a local factor with no underlying thrombophilia and in 35 patients (39.3%) neither thrombophilia nor local factor were identified and were considered idiopathic.

Antithrombin deficiency had been, in the initial clinical screening, ruled out in all patients based on normal AT anti-FXa activity (or a reduction in anti-FXa activity in the setting of similar reduction in other liver synthesized factors) and the lack of familial history of neither thrombosis nor AT deficiency. The median values of AT activity measured as anti-FXa activity were 86.8% ± 17%. Of note however, in this cohort there were 25 patients who presented levels of AT below 80% (median 68%, range: 48-79), which isolated could had been indeed considered AT deficiency. Nevertheless, these patients had a concomitant decrease in other coagulant factors such as protein C (median 72%, range: 33-99), protein S (median 82%, range: 68-130), FII (median 77%, range: 57-92) or FX (median 73%, range: 44-143). Therefore, these decreased AT levels were considered acquired and clinically not relevant, assuming that they were due to either consumption or to a global decrease in liver factors synthesis.

Using the biobank samples of these 89 cases, AT function was reassessed by measuring both functional activity (by anti-FXa and anti-FIIa tests) and by immunological methods (Laurell and western blot). Similar to what had been found in the clinical setting, this secondary screening showed that 21 cases had low AT levels in at least two tests (either AT anti-FXa, anti-FIIa activity or the two immunological assays). In these 21 cases with two positive findings

| TABLE 1 | Baseline characteristics and etiological SVT factors of Cohort 1 |
|-----------------|-----------------|-----------------|
|                | PVT (n = 68)    | BCS (n = 21)    | SVT (PVT + BCS) n = 89 |
| Baseline characteristics n (%) or mean ± SD |                 |                 |                          |
| Age at diagnosis (y) | 44 ± 21         | 39 ± 12         | 43 ± 20                  |
| Men (%)            | 44 (65)         | 6 (29)          | 50 (56%)                 |
| Haemoglobin (g/dL) | 128 ± 23        | 133 ± 27        | 129 ± 23                 |
| Platelet count (×10^9/L) | 213 ± 127      | 285 ± 137       | 231 ± 132                |
| ALT (U/L)          | 47 ± 48         | 105 ± 133       | 61 ± 81                  |
| AST (U/L)          | 40 ± 41         | 106 ± 184       | 56 ± 100                 |
| FA (U/L)           | 292 ± 307       | 435 ± 240       | 326 ± 297                |
| GGT (U/L)          | 123 ± 167       | 182 ± 123       | 137 ± 159                |
| Bilirubin (mg/dL)  | 1 ± 2           | 3 ± 2           | 2 ± 2                    |
| Albumin (g/L)      | 39 ± 8          | 33 ± 6          | 37 ± 8                   |
| Prothrombin time (%) | 79 ± 19        | 61 ± 16         | 75 ± 20                  |
| Ascites            | 8 (12)          | 17 (81)         | 25 (28)                  |
| Etiological factors |                 |                 |                          |
| Idiopathic         | 28 (41)         | 7 (33)          | 35 (39)                  |
| Thrombophilia      | 15 (22)         | 13 (62)         | 28 (31)                  |
| Myeloproliferative neoplasm | 8 (12)      | 8 (38)          | 16 (18)                  |
| Behcet             | 0               | 1 (5)           | 1 (1)                    |
| Paroxysmal hemoglobinuria | 0            | 1 (5)           | 1 (1)                    |
| Antiphospholipid syndrome | 1 (2)      | 2 (10)          | 3 (3)                    |
| Protein C deficiency | 3 (4)          | 1 (5)           | 4 (4)                    |
| Prothrombin mutation G20210A | 3 (44)     | 1 (5)           | 4 (4)                    |
| Local factor       | 25 (37)         | 1 (5)           | 26 (29)                  |
| Splenectomy        | 2 (3)           | 0               | 2 (0)                    |
| Omphalitis         | 1 (2)           | 0               | 1 (0)                    |
| Hepatic abscess    | 1 (2)           | 0               | 1 (0)                    |
| Pancreatitis       | 7 (10)          | 0               | 7 (8)                    |
| Inflammatory bowel disease | 0           | 1 (4.8)         | 1 (1)                    |
| Abdominal surgery  | 14 (20)         | 0               | 14 (15)                  |

Abbreviations: BCS, Budd Chiari syndrome; SVT, splanchnic vein thrombosis; PVT: portal vein thrombosis.
suggesting AT deficiency (even if they also had decreased levels of protein C or protein S), SERPINC1 was sequenced and a genetic variation was found in 2 cases:

- Patient 1 was a 48-year-old woman that presented a PVT without any associated local inflammatory process. During the PVT work-up she was diagnosed with JAK2 myeloproliferative neoplasm (MPN) and therefore anticoagulation was initiated. The remaining tests of the etiological study were considered normal. Despite the anticoagulation treatment this patient presented a more aggressive course with progression of PVT. Besides MPN, in the present analysis a mild AT deficiency was detected based on her anti-FXa activity of 70%. Moderately reduced levels of AT in plasma were verified by Laurell (84%) and Western blot, while in the genetic analysis a synonymous change affecting SERPINC1 in heterozygosis, c.468>T; p.Ala156Ala, was identified. This genetic defect has been described as rs759369437 and has a minor allele frequency (MAF) of 0.00002 (data from Exome Aggregation Consortium Database, ExAC). Although there are synonymous mutations with pathogenic effect that may contribute to different disorders, we performed a familial study (Figure S2) that suggested that this SERPINC1 synonymous mutation might not have a pathogenic effect.

- Patient 2 was a 60-year-old man with biliary pancreatitis that developed a splenic thrombosis. The thrombophilia study showed AT deficiency by functional methods (anti-FXa 65% and anti-FIIa 70%) but as the Protein S levels were also slightly diminished (60%) the clinical interpretation had been that there was a global diminishment of coagulant factors secondary to reduced liver synthesis. Indeed, antigen levels revealed nearly normal AT levels in the biobank sample detected by Laurell (91%) and Western blot after SDS-PAGE (Figure 1). However, an abnormal AT form was detected in native gels but with no increased levels of the latent form (Figure 1). Crossed immunoelectrophoresis in the presence of heparin confirmed the type II deficiency without increase in AT forms with low heparin affinity (Figure 1). Sequencing of SERPINC1 revealed a heterozygous missense mutation in exon 3 c.595G>A; p.Gly199Arg. The mutation affects a residue not conserved among the serpin superfamily located at the loop connecting the strand s1A and the helix E (Figure 1). This mutation, also reported as rs778341415, has a very low MAF = 0.00003 in ExAC, and is reported as a variant with uncertain significance at ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/variation/432933/). This mutation is not included in the HGMD database (http://www.hgmd.cf.ac.uk/ac/all.php). Interestingly, the residue is highly conserved (80%) in ATs from 49 species (Figure 1). All these data support that this mutation caused a type II deficiency with impaired reactivity (type II RS). No long-term anticoagulation was administered and no re-thrombosis was observed after 9 years of follow-up.

Afterwards, since mutations that are not usually detected by current functional assays are usually located in exons 2 and 7, these exons were sequenced in the whole cohort of 89 patients. One mutation was found:

- Patient 3 was a 51-year-old woman with a known MPN that during follow-up developed a portal and splenic vein thrombosis. She presented strictly normal levels of AT anti-FXa (96%) and anti-FIIa (99%) and no AT deficiency according to immunological assays. Since the development of thrombosis she was on anticoagulation and has not presented new thrombotic events after 7 years of follow-up. The present study, however, has enabled to detect that she was a heterozygous carrier of the c.89T>A; p.Val30Glu mutation (AT Dublin). The presence of this mutation was confirmed in a new blood sample.

Finally, in all 89 patients, analysis of plasma AT by western blot by using native and denaturing electrophoretic conditions, enabled the detection of a patient with N-hypoglycosylation defect:

- Patient 4 was a 63-year-old man with chronic pancreatitis as a result of CFTR gene mutation and chronic alcohol consumption. In this setting, he presented with an acute PVT. The thrombophilia study was negative (including AT anti-FXa levels of 94% and anti-FIIa 89%) and it was assumed that the PVT was secondary to local inflammation and pancreatitis. Anticoagulation was administered for 6 months. However, 2 years later the patient presented an acute pulmonary thromboembolism requiring resuming of anticoagulation. The current study displayed increased levels of N-hypoglycosylated forms of AT, which was verified by increased levels of N-hypoglycosylated forms of α1-antitrypsin and transferrin (Figure 2).

Summing up, overall four patients (4.5%) in whom AT deficiency had been previously clinically discarded, in the present study were found to have different AT mutations or deficiencies. Table 2 summarizes the more clinically relevant findings.

**Cohort 2** Report of congenital antithrombin mutations and defects associated to SVT in a large cohort of patients with AT deficiency.

Two hundred seventy-one patients with confirmed congenital AT deficiency were included in this cohort. Among them, 166 cases had type II AT deficiency and 105 had type I AT deficiency.

Splanchnic vein thromboses was reported in 11 of the 271 patients (4.1%). In all cases, SVT was the first thrombotic event and appeared in patients that were not previously on anticoagulation. Table 3 shows the characteristics and genetic defects of these patients. Four of the gene defects described in this study are being reported for the first time. Nine cases of 11 (81.8%) had type I deficiency caused by a severe genetic defect. We point 2 families (with 2 cases each) with type I deficiency and mesenteric thrombosis: one family presented a deletion affecting exon 4 and the other one a 31 bp deletion affecting the splicing signal and first 29 nucleotides
FIGURE 1  Characteristics of the p.Gly199Arg mutation identified in a patient with splanchnic vein thrombosis and antithrombin deficiency. A, Western blot of plasma antithrombin after native electrophoresis. Native antithrombin is pointed by a black arrow, while the variant antithrombin is pointed by a red arrow. B, Crossed immunoelectrophoresis in presence of heparin. The plasma antithrombin forms with low (L) and high (H) heparin affinity of a healthy control, the proband and a patient with antithrombin deficiency caused by a type II heparin-binding site mutation (AT Toyama; p.Arg79Cys) is shown. C, Localization of the Gly199 residue (red) in the structure of antithrombin. The reactive centre loop is marked in green. D, Alignment of antithrombin from 49 species at the region of interest, visualized by Jalview. Gly199 is pointed by an arrow in red.
of exon 7. Of note, only one among the 166 patients with type II deficiency presented SVT. In this cohort, 1 patient had AT deficiency because of a congenital glycosylation disorder (Table 3).

4 | DISCUSSION

The present study is the first to evaluate the incidence of undetected AT deficiencies among patients with SVT in whom a first initial screening had ruled out AT disorders. Moreover, the study of a large cohort of patients with AT deficiency has revealed new information on the role of this strong natural anticoagulant in SVT.

The analysis of Cohort 1 shows that, currently, clinical assessment of AT function is mainly based on anti-FXa activity. Anti-FXa activity should adequately reflect the presence of both diminished levels of AT or impaired function, but the presence of the recently reported mutations causing transient deficiencies would be unnoticed by this method. More importantly however, we want to remark that sometimes it can be difficult to establish strict cut-off values of anti-FXa activity levels as there are many external agents that can affect its value, thus rendering...
TABLE 2  Antithrombin deficiencies found in Cohort 1, 89 patients with SVT in which antithrombin deficiency was ruled out in the first screening

| Anti-FXa | Anti-FIIa | Prot C | Prot S | SVT Aetiology     | AT deficiency | SERPINC1 variant | Follow-up         |
|---------|---------|-------|-------|------------------|---------------|------------------|-------------------|
| 65%     | 70%     | 105%  | 61%   | Local factor     | Type II deficiency | c.595G>A (p.Gly199Arg) | No anticoagulation |
| 96%     | 99%     | 77%   | 60%   | Myeloproliferative neoplasm | Transient deficiency | c.897T>A (p.Val30Glu) | Anticoagulation |
| 94%     | 89%     | 99%   | 130%  | Local factor     | N-Hypoglycosylation |               | Re-thrombosis     |

Abbreviations: Anti-FIIa, anti-Factor II activated; Anti-FXa, anti-Factor X activated; Prot C, Protein C; Prot S, protein S; SVT, splanchnic vein thrombosis.

TABLE 3  Genetic and clinical features of patients with antithrombin deficiency suffering from splanchnic vein thrombosis

| Patient | Anti-FXa | SERPINC1 defect | Deficiency |
|---------|---------|----------------|------------|
| 1       | 54%     | c.236G>A; p.Arg79His (CM890016) | Type II HBS |
| 2       | 50%     | c.1366G>C; p.Gly456Arg (CM940138) | Type I |
| 3       | 52%     | c.481C>T; p.Arg161Ter (CM7900156) | Type I |
| 4       | 40%     | c.495del; p.Ala166Profs*7 (New gene defect) | Type I |
| 5       | 51%     | c.490del; p.Arg164Aspfs*9 (New gene defect) | Type I |
| 6       | 40%     | c.814dup; p.Tyr272Leufs*3 (CM100156) | Type I |
| 7       | 37%     | c.1219-1_1248del; p.Val407_Ala414del (New gene defect) | Type I |
| 8       | 60%     | c.398A>C; p.Glu133Pro (CM940132) | Type I |
| 9       | 50%     | Exon 4 deletion (New gene defect) | Type I |
| 10      | 52%     | Exon 4 deletion (New gene defect) | Type I |
| 11      | 63%-91% | No SERPINC1 defect | Congenital disorder of glycosylation |

Abbreviations: Anti-FXa: anti-factor X activated; HBS, heparin-binding site.

Based on the recent discovery of mutations causing transient AT deficiency and the finding of N-glycosylation related AT deficiencies, as well as the doubtful accuracy and reliability of AT anti-FXa activity as a diagnostic test, we hypothesized that these new diagnostic techniques could play a role in facilitating the diagnosis of masked AT deficiencies in the setting of SVT. In Cohort 1 we identified that 4.5% of patients previously considered to have a normal AT function presented an underlying AT disorder, which is especially significant when contrasted with the general population prevalence of AT deficiency of 1/500-5000. Importantly, in all these four patients the development of SVT had been exclusively attributed to either a concomitant local inflammation or to a MPN, but it was afterwards proved that they also had an AT disorder. These findings reinforce the concept that having two synergic prothrombotic conditions probably leads to an even higher hypercoagulable state, and that therefore a complete etiologic work-up should be performed in all patients, even if they already present a plausible explanation for the thrombotic event.

The analysis of Cohort 2, one of the largest worldwide cohort of patients with AT deficiency and a previous thrombotic event, demonstrates that thrombotic events in the portal and mesenteric veins is not an unusual localization among carriers of this severe thrombophilia. Indeed, in 41% of AT deficiency cases, the first thrombotic event was in the splanchnic territory. Although it is highly relevant if we take into account that the general population prevalence of SVT is <5 in 10 000 inhabitants, it is not possible to infer from this cohort the incidence of SVT among patients with AT, as they were only diagnosed with AT deficiency after they had already developed SVT. The molecular analysis revealed that this localization is mainly restricted to cases with genetic defects causing severe AT deficiency, as most cases with SVT and AT deficiency had type I deficiency. Indeed, the percentage of patients with type I deficiency that developed SVT was significantly high: 9/105 (8.6%). Our study identified four new SERPINC1 defects not previously described in patients with SVT, all causing type I deficiency. Three by causing frameshift and generating a premature stop codon: c.495del (p.Ala166Profs*7), p.Arg164Aspfs*9, and c.1219-1_1248del (p.Val407_Ala414del); and the fourth by a structural variation (deletion of exon 4). Moreover, we point out two of these new SERPINC1 gene defects (deletion of exon 4 and c.1219-1_1248del (p.Val407_Ala414del) that affected them difficult to interpret: functional analysis of AT may identify cases with low anti-FXa activity levels that are not due to congenital deficiency. Impaired hepatic synthesis or coagulopathy caused by thrombin generation in the setting of acute thrombosis may lead to the detection of falsely low levels of AT, which are frequently accompanied by simultaneous low levels of other anticoagulant factors and that therefore are not considered to be clinically relevant in relation to risk of rethrombosis or need of treatment with long-term anticoagulation.
two members of two different families developing SVT. On the other hand, only one case out of 166 cases with type II deficiency developed SVT, suggesting that this patient could have presented an additional undetected risk factor favouring the development of this severe thrombotic event.

Finally, disorders affecting the efficacy of the N-glycosylation can impair AT function and therefore increase the risk of thrombosis.

In this study we have identified two cases with SVT due to hypoglycosylation, one in each cohort. This finding strongly supports that this aberrant post-translational modification causes a severe pro-thrombotic state.

We must acknowledge that one of our main limitations is the difficulty in differentiating the new mutations described in the present study from mere polymorphisms. However, taking into account the clinical setting of thrombosis and the low frequency in general population (MAF) of the mutations reported that reinforces their pathogenic role, it seems reasonable to assume causality between our findings and the development of thrombosis.

According to current evidence, it is recommended to screen patients with SVT for underlying thrombophilia and, given its high frequency among SVT, AT should be carefully studied. Figure 3 suggests an AT screening algorithm depicting when to sequence SERPINC1 and to assess N-glycosylation defects. Even though there is not enough evidence nor cost-effectiveness studies to suggest systematic screening of SERPINC1 mutations and CDG in all SVT patients (instead of performing only anti-FXa activity determinations), we consider that the findings of the present study, together with the small size of SERPINC1 and the development of massive sequencing methods that allow a relatively easy, fast and cheap sequencing of this gene, make it appropriate to recommend a comprehensive AT evaluation in cases of rethrombosis or doubtful interpretation of anti-FXa activity levels (Figure 3).

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* In these cases it would be also interesting to sequence SERPINC1 and screen for CDG. However, as it is probably not cost-effective, more studies are warranted before recommending this attitude in clinical practice
** Consider sequencing SERPINC1 and screen for CDG to further characterize the type of AT deficiency

**FIGURE 3** Proposed algorithm for antithrombin (AT) screening in patients with splanchnic vein thromboses (SVT)
CONFLICT OF INTEREST
VHG and JCGP have received research grants from Gore. The remaining authors declare no conflicts of interests.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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