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Received: 27 June 2024 | Revised: 3 September 2024 | Accepted: 1 October 2024

https://doi.org/10.1016/j.jtha.2024.10.005

ORIGINAL ARTICLE

Novel insights into antithrombin deficiency enabled by mass spectrometry-based precision diagnostics

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Funding Information
This project received funding from the European Union's Horizon 2020 Research and Innovation program under the Marie Skłodowska-Curie grant agreement (843615), Fundación Séneca (21886/PI/22), and the Instituto de Salud Carlos III (ISCIII) through project PI21/00174 co-funded by the European Union. MEM-B has a Ramon y Cajal contract (RYC2021-031000-I, Ministerio de Ciencia, Spain).

Abstract

Background: Although P5 (preventive, personalized, predictive, participatory, psycho-cognitive) medicine and patient-focused healthcare are gaining ground in various healthcare areas, the diagnosis of antithrombin deficiency (ATD) is still based on crude diagnostic tests, clustering patients into clinically heterogeneous subgroups whereby relevant thrombophilia phenotypes may go unnoticed. Clinical pathways and the majority of evidence are based on these tests; therefore, generic treatment is still the norm.

Objectives: To unravel the heterogeneity of ATD, a mass spectrometry (liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry [LC-MRM-MS])-based test for antithrombin was developed allowing molecular characterization of the antithrombin proteoforms in patient plasma. This study provides the first insight into the tests' clinical performance.

Methods: Plasma from 91 unrelated ATD patients and 41 patients with a congenital disorder of glycosylation affecting antithrombin glycosylation were characterized functionally, genetically, and analyzed by LC-MRM-MS. An established data analysis strategy was applied for quantitation and molecular characterization of antithrombin proteoforms.

Results: The test recognized patients with a quantitative defect, discriminated between type I and type II ATD, and identified variant proteoforms. Overall, the diagnostic sensitivity for ATD was 100% for LC-MRM-MS compared with 81.1% by the functional test. Type II ATD, a subtype prone to misdiagnosis, revealed an even larger difference of 100% identification by LC-MRM-MS vs 56.8% by functional test.

Conclusion: The qualitative and quantitative mass spectrometry–based AT-test can serve as a platform for investigating the molecular basis of the clinical heterogeneity of ATD. This "precision diagnostics" approach for ATD can lower diagnostic uncertainty and modernize the ATD diagnostic and clinical pathways.

KEYWORDS
hereditary antithrombin deficiency, mass spectrometry, molecular testing, protein isoforms

Manuscript handled by: Alan Mast

Final decision: Alan Mast, 1 October 2024

1 | INTRODUCTION

Antithrombin deficiency (ATD) is a clinically heterogenous disorder due to the large number of possible genetic and posttranslational modifications (PTMs) influencing the expression, secretion, functionality, and stability of antithrombin (AT), a key coagulant serpin [1–4]. Although AT was first mentioned in 1939, and the first notion of ATD was in 1965, current diagnostic tests still lack sensitivity and specificity leading to missed diagnoses and cannot distinguish patients at high and low risk for venous thromboembolism (VTE) [5–7]. Without anticoagulant treatment, ATD leads to a high annual risk of recurrent venous thromboembolism (VTE) of 8.8% [8]. Consequently, it is suggested to treat ATD patients indefinitely with an anticoagulant, such as vitamin K antagonist drugs or direct oral anticoagulants (DOACs) [9–11]. Such treatment brings along bleeding risk which, although seemingly low at an annual risk of 0.5% to 0.8%, contributes to morbidity while the annual VTE recurrence risk is still 2.7%. The current clinical care pathway for ATD, suffering from the unresolved clinical heterogeneity of ATD and a one-size-fits-all approach, leads to over- and undertreatment of individual ATD patients and deserves modernization.

To reduce diagnostic uncertainty in the ATD clinical pathway and facilitate P5 medicine (preventive, personalized, predictive, participatory, psychocognitive), a better understanding of the spectrum of AT proteoforms and their pathological diversity is key [12]. The current diagnostic tests for ATD do not meet the required clinical performance to distinguish between low and high-risk ATD impeding personalized treatment. Commercially available AT activity and antigen tests only allow the classification of patients into 2 subtypes, type I (quantitative) and type II (qualitative) ATD with varying clinical severity [13], and lack sensitivity for specific types of ATD, resulting in missed diagnoses [14–16]. Genetic testing offers specific insight into the exact molecular defect. However, it does not identify defects caused by PTMs, such as N-glycosylation, and only provides a blueprint of how the protein may be expressed. Importantly, N-glycosylation may have key functional and clinical relevance [15]. This highlights that clinical phenotypes are only indirectly caused by genes, and instead proteins, playing a direct role in diseases, are likely to provide additional information [17,18]. Therefore, molecular information on the proteoforms present in ATD patients may hold the key to better identify and characterize ATD patients.

Molecularly, ATD is a complex disorder. Type I ATD is caused by genetic variants impeding the translation and/or secretion of the mutant proteoform resulting in a decrease in AT concentration of approximately 50% (with the remaining AT originating from the wild-type allele) [19]. In contrast, type II ATD or ATD caused by aberrant glycosylation leads to the presence of variant AT proteoforms in the circulation with varying clinical severity and challenging diagnoses due to the unpredictable influence of the variant proteoforms on the detection by AT activity tests [13,14,16,20]. Current diagnostic tests focus on the activity or concentration of the total AT pool (Figure 1) obtained through indirect approaches, eg, based on chromogenic or latex agglutination tests. It is therefore not surprising that pleas have been made to introduce refined molecular tests for AT, aiming to fully characterize, quantitatively and qualitatively, AT proteoforms in plasma [20–22]. Ideally, the envisioned molecular test(s) should incorporate all molecular features of AT including glycosylation.

Recently, a test based on liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry (LC-MRM-MS) was developed [23] for the molecular characterization of plasma AT proteoforms. In contrast to current tests used in the diagnosis of ATD, MS enables direct monitoring of the AT proteoforms present in a patient sample allowing both quantitation (in μmol/L) and (in theory) molecular characterization (Figure 1) [24]. The test was analytically validated, which verified the tests' ability to quantify the AT concentration of clinical samples in clinically relevant ranges, and a case report indicated that the theorized molecular characterization is feasible [25]. However, to assess the potential of the LC-MRM-MS-based AT-test for improving patient management, its clinical performance must be evaluated.

Clinical performance studies ideally require a prospective setup in which patients with suspected clinical symptoms for (in this case) ATD are included and tested with both the current and the comparator test [26]. Due to the low frequency of ATD in the VTE population in combination with the lack of experience in the ability of the test to identify mutations, it was decided to first assess the tests' scientific validity retrospectively in a hereditary ATD patient cohort. Moreover, ATD has also been reported in patients with type 1 congenital disorders of glycosylation (CDG), who suffer from a systemic N-glycosylation defect leading to a varying degree of AT hypoglycosylation. To assess the tests' ability to detect alterations in AT glycosylation, a cohort of patients with CDG was also evaluated. Notably, the clinical care pathway for CDG, and therefore the clinical need, is different in CDG patients compared with ATD patients. The aim of this study was to assess the ability of the test to identify ATD, stratify between type I and II ATD, and investigate to what extent the test enables full molecular characterization of the AT proteoforms present in clinical samples.

2 | METHODS

2.1 | Patient samples

Samples from 132 unrelated patients with genetically confirmed ATD or CDG, of which the majority had a history of thrombosis, were selected for LC-MRM-MS analysis [16,27]. Samples were processed within 24 hours of blood draw by centrifuging citrate tubes (2200 *g*, 20 minutes), collecting the platelet-poor plasma, and storing them at −80 °C. All patients gave informed consent following ethical guidelines, as approved by the institutional review board of the Hospital Universitario Morales Meseguer and in accordance with the Declaration of Helsinki of 1964 and its subsequent amendments.

Samples from hereditary ATD patients (N = 91) were selected from a biobank of the Hospital Universitario Morales Meseguer, which contains over 350 unrelated cases recruited between 1998 and 2023.

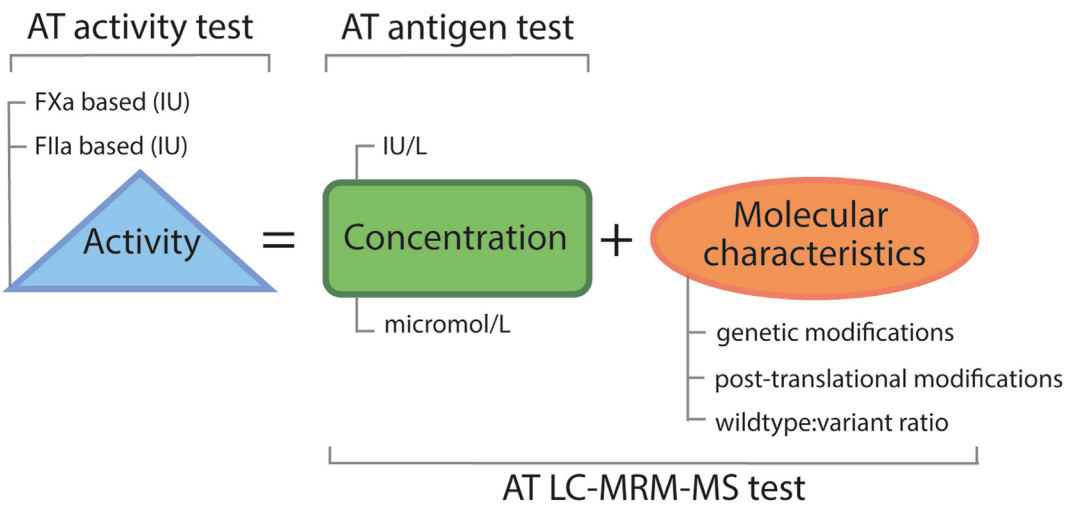


FIGURE 1 Focus of contemporary (activity and antigen test) and new tests (LC-MRM-MS) for ATD.

Patients underwent thrombophilia screening and were recruited via 3 main routes: 1) due to a clinical event (eg, VTE, *N* = 77); 2) screening indicated by family studies (*n* = 9, note that only unrelated samples were analyzed for the study described here); 3) screening in a gynecologic setting or when prescribed contraceptives (*n* = 5). Selection aimed to include a varying repertoire of mutations that were genetically confirmed to carry a pathogenic genetic variant in the AT gene *SERPINC1*. Most patients enrolled in this study have maintained AT deficiency and thrombosis, which are explained by *SERPINC1* variants. These cases are easily diagnosed by functional and genetic methods. However, we also identified cases with much more complex diagnoses, as they have transient AT deficiency explained by different mechanisms, from structural constraints resulting in pathogenicity only under stress conditions to the use of different functional methods that may not detect the functional consequences of the *SERPINC1* variant. The characteristics of these cases have been published [16]. Thus, we systematically sequenced the *SERPINC1* gene in all 350 cases with suspicion of AT deficiency recruited during more than 25 years, and selected samples of cases carrying different *SERPINC1* variants independently of having normal AT levels. Twelve *SERPINC1* variants selected for this study caused transient AT deficiency, and the samples evaluated in this study had no AT deficiency caused by the variant (p.Val30Glu, p.Arg45Trp, p.Pro73Leu, p.Arg79His, p.Val137Ala, p.Arg177Cys, p.Gly199Arg, p.Asn224His, p.Glu227Lys, p.Ala416Ser, p.Ser426Leu, and p.Pro439Thr).

To assess the potential of the LC-MRM-MS test to identify PTMs, patients with CDG (*N* = 41) were also included. CDG leads to systemic changes in the glycosylation, affecting AT as well as other proteins, and transferrin glycoforms were analyzed by high-performance liquid chromatography to ascertain the diagnosis of CDG (which is the gold standard for CDG diagnostics, although diagnosis is still challenging [28]).

For comparative purposes, samples from 37 healthy controls were also analyzed. These controls were recruited via the Leiden University Medical Center Voluntary Donor Service (as approved by the Leiden University Medical Center ethics board) which recruits (apparently) healthy hospital employees to donate blood for studies.

2.2 | Genetic analysis

Genetic defects were identified by next-generation sequencing of the *SERPINC1* gene, or 72 genes potentially involved in CDG [29,30]. For *SERPINC1*, the exons, flanking regions, and 1500 bp of the promotor region were amplified by polymerase chain reactions (PCR, Expand Long Template Polymerase) and sequenced with ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit on a 3130×l Genetic Analyzer (Applied Biosystems). For detailed information, see de la Morena-Barrio et al. (2012) [31]. Obtained sequences were compared with a reference sequence (GenBank NG_012462.1) using SeqScape v2.5 Software (Applied Biosystems). Of note, gross deletions were analyzed by multiplex ligation-dependent probe amplification (MLPA) using the *SALSA MLPA* Kit P227 SerpinC1 (MRC-Holland) [32]. For CDG patients, exons and flanking regions of the *PMM2* gene were PCR amplified and sequenced. If no mutation was found, whole exome sequencing was performed on an Ion Proton platform (Life Technologies) using the AmpliSeq kit and Ion Reporter Software. Results were validated using corresponding primers [30]. Most CDG patients (*N* = 37) were subtyped as PMM2-CDGs, with the remaining 4 patients carrying other subtypes (DPAGT1-CDG, MPI-CDG, ALG12-CDG, and SSR4-CDG).

2.3 | AT activity

AT activity was measured in all samples except one (*n* = 131) using an in-house developed method based on a chromogenic antiactivated factor (F)Xa method (HemosIL Liquid AT, #0020008900, Werfen) using S-2765 substrate and bovine FXa read-out on a Synergy HT plate reader. The method was verified toward an automated

coagulometer (ACL-TOP), using a chromogenic substrate (#0020008910, Werfen) and bovine FXa (#0020008920, Werfen). Calibration was performed by serial dilution of a reference plasma generated by pooling 100 healthy blood donors. Normal ranges, as tested in 250 healthy blood donors, are 80% to 120%.

2.4 | LC-MRM-MS test

The LC-MRM-MS test was developed and analytically validated according to established laboratory guidelines, as described elsewhere [23]. Patient samples were analyzed in duplicate, whereas controls were analyzed in duplicate (N = 11) or singlicate (N = 26). Upon suspicion of a variant proteoform in a sample, the presence of the proteoform was confirmed as described elsewhere (Kruijt et al., manuscript submitted). A system suitability test sample was measured fivefold before and after each experimental batch to monitor system performance. Furthermore, 2 quality control (QC) samples were included threefold in each experimental batch to monitor the total test performance. The 2 QCs showed means ± coefficient of variation of 1.37 μmol/L ± 6.0% and 1.18 μmol/L ± 5.9% (based on quantitative peptide LVSAN). Additionally, ion ratios were evaluated for the total data set to ensure data quality and exclude interferences.

2.5 | Data analysis

Raw MS files were automatically interpreted using Agilent MassHunter Workstation Quantitative Analysis (v10.0), followed by manual inspection and adjustment if necessary. Results were imported as Excel files into R studio (v1.4.1717). All data analysis steps (QC, data verification, and exploration and reporting) were executed by in-house-built scripts apart from proteoform verification, which was examined manually. In-depth information and examples of the data analysis process are found elsewhere (Supplementary Material and Kruijt et al., manuscript submitted).

TABLE 1 Summary characteristics of ATD cohorts studied by liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry.

Characteristics	Patients (N, %)	Activity (%)	Thrombosis reported (%)[a]
Hereditary ATD	91	33-102	
Type I	53 (58.2)	33-88	90.6
Type II	38 (41.8)	40-102	58.8[b]
Subtype unknown	10 (11.0)	40-97	100[b]
HBS	8 (8.8)	44-90	50.0
RS	9 (9.9)	50-96	55.5
PE	11 (12.1)	46-102	45.5
CDG	41	9-116	NA
Controls	37	ND	NA

ATD, antithrombin deficiency; CDG, congenital disorders of glycosylation; HBS, heparin-binding site; PE, pleiotropic effect; RS, reactive site; NA, not available; ND, not determined.
[a] Thrombosis reported entails clinically confirmed deep vein thrombosis, PE, cerebral venous thrombosis, or mesenteric vein thrombosis. Note that there was one type I sample with activity >80%, as this was a sample harboring a transient type I ATD mutation.
[b] Four samples did not have a complete clinical background.

3 | RESULTS

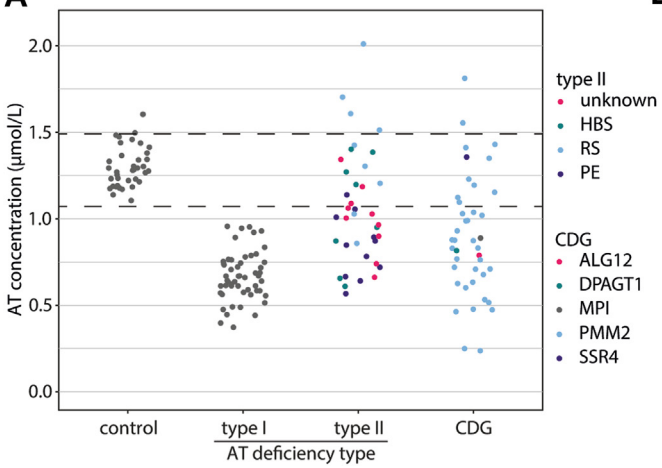
The LC-MRM-MS test was applied in a cohort of genetically confirmed ATD patients (n = 91, see Table 1 for patient details). The majority of ATD patients had clinical symptoms, although asymptomatic ATD patients were also included, originating from family studies or gynecologic thrombophilia screenings. As PTMs are also known to affect AT function and are included in the LC-MRM-MS test, a cohort of CDG patients (N = 41) was also analyzed [15]. These patients had a systemic defect in their N-glycosylation which, among other proteins, affects the glycosylation of AT. CDGs are diagnosed via a different diagnostic pathway than hereditary ATD, in general by a pediatrician, with multiple clinical effects in these patients. Thrombophilia is not a hallmark feature of CDG patients, although thrombosis occurs more often in CDG patients than in the general population and therefore frequent thrombophilia screening is recommended (eg, yearly or before surgery/during pregnancy) [33,34].

The LC-MRM-MS test quantifies the concentration of 23 AT peptides, from which the overall AT concentration (based on a quantifier peptide), as well as in-depth information on the status of each protein stretch (based on qualifier peptides), can be derived (Kruijt et al., manuscript submitted). The anticipated output of the LC-MRM-MS test per patient was 1) the AT concentration in μmol/L and 2) the presence of molecular defects either caused by mutations or variations in the glycosylation. Together this would allow the identification of ATD and subtype classification.

3.1 | AT concentration

A quantitative deficiency resulting in low AT concentrations is a hallmark of ATD, specifically for type I ATD. To this end, the concentration of the total AT proteoform pool was analyzed by LC-MRM-MS. All control samples showed concentrations within the reference intervals (RIs; 1.07-1.49 μmol/L), apart from 2 samples that were slightly higher than the RI (Figure 2). As expected, all patients carrying a type I ATD had a low AT concentration ranging between 0.37 and 0.96 μmol/L. Type II ATD, usually defined by low AT activity levels but normal antigen levels, showed more heterogeneous concentrations ranging between 0.57 and 2.01 μmol/L. Of the 38 type II ATD patients, 23 patients had an AT concentration below the RI and 11 patients had a concentration within the RI. Interestingly, 4 type II ATD patients presented with concentrations above the RI, all harboring a mutation

A



B

Cohort	Subtype	Antithrombin concentration Low	Normal	High
Control	-	-	35	2
ATD	All	76	11	4
	Type I	53	-	-
	Type II	23	11	4
	Subtype unknown	7	3	-
	HBS	4	4	-
	RS	2	3	4
	PE	10	1	-
CDG	All	30	9	2
	ALG12	1	-	-
	DPAGT1	1	-	-
	MPI	1	-	-
	PMM2	27	8	2
	SSR4	-	1	-

FIGURE 2 Quantitative analysis of AT in plasma of control, ATD and CDG samples by LC-MRM-MS. A) Concentrations of the analyzed samples, grouped per type. Dashed lines indicate reference intervals. Subtypes are specified for type II and CDG samples as indicated in the legend. **B)** Overview of the number of samples that are low, normal or high in concentration based on previously established reference intervals, grouped per type and subtype.

in either Arginine-425 or Serine-426, the reactive site of AT. Samples from CDG patients showed a wide concentration range, between 0.24 and 1.81 µmol/L, with 30 patients presenting with low concentrations, 9 with normal concentrations, and 2 patients with elevated concentrations. These results highlight the variability in AT concentrations found in type II ATD and CDG patients. Taken together, 76/91 (83.5%) of ATD patients and 30/41 (73.2%) of CDG patients could be identified as ATD based on the quantitative information of the LC-MRM-MS test.

3.2 | Molecular characterization

The qual/quant ratio, calculated by dividing the concentration of a qualifier peptide over the overall AT quantity, indicates how well an individual peptide concentration agrees with the overall AT concentration. A maximum deviation from the qual/quant ratio was established based on previously obtained precision data and empirical data (see also Supplementary Material and [23]). Deviating qual/quant ratios are indicative of the presence of varying molecular proteoforms in a single sample. It was anticipated that deviating qual/quant ratios would only occur for type II ATD, as type I ATD mutations are believed to not be expressed and/or secreted. Indeed, deviating qual/quant ratios corresponding to the anticipated affected peptide (as based on genetic information) only occurred in 24 out of 38 (63.2 %) type II ATD samples; these patients likely expressed a variant AT proteoform (Table 2) [35].

For these 24 samples, the amount of wild-type proteoform that is present could be calculated based on the ratio of the total AT: $\%\ variant\ proteoform = (1 - qual/quant\ ratio) \times 100\%$, revealing proteoforms to be present at levels between 16% and 58%. Of the 38 type II ATD samples, there were 3 mutation sites that each had 3 affected samples; namely Asn-225 (p.Asn224His), Glu-227 (p.Glu227Lys), and Arg-425 (p.Arg425del, p.Arg425His, p.Arg425Cys). These sites had mean qual/quant ratios ± SD of 1.35 ± 0.08, 0.72 ± 0.04, and 0.52 ± 0.07, respectively, indicating that (similar) mutations have a relatively consistent effect on the ratio of variant:wild-type proteoforms.

Of the 14 type II ATD samples that did not show deviating qual/quant ratios, 9 samples harbored mutations outside of the assay coverage. Interestingly, 12 out of 14 unidentified samples were already flagged as being ATD, based on their low concentration. The 5 samples that had mutations occurring in the monitored wild-type peptides also had low concentrations, likely caused by a low expression and secretion of the variant proteoform, hampering their identification by the qual/quant ratio.

3.3 | Identification of specific proteoforms by variant peptides

To confirm the identity of the specific molecular variant, a confirmative approach was developed in which the MS test was adjusted to measure variant peptides. In this pilot study, the genetic information available for each patient eased the identification of the variant peptide. However, in future investigations, the established variant peptide transitions may be directly applied without prior genetic knowledge or possible variants may be extracted from literature (as was previously applied in a case report [25]). Variant peptides could be identified in 22 ATD patients amounting to a total of 19 unique variant peptides (Table 2). The approach was applied to all samples, including those that were not indicated to have a qualitative defect based on the qual/quant ratio and type I ATD patients. Using this approach, 10 patients who were not identified using the qual/quant ratio were found to carry a variant proteoform. When combined, the information from the qual/quant ratios and the variant peptide transitions

TABLE 2 Identification of qualitative deficiencies and variant proteoforms.

Patient	Mutation[a]	Deficiency subtype	Qual/quant ratio or glyco/quant ratio (Peptide)[b]	Variant peptide detected[b]
1	p.Val30Glu	unknown[c]	0.49 (HGSPV)	Yes
2	p.Val30Glu & p.Arg425Cys	unknown[c] type II RS	0.69 (HGSPV) 0.58 (SLNPN)	Yes
3	p.Arg45Trp	type II HBS	0.49 (HGSPV) 0.47 (DIPMN)	No
4	p.Pro73Leu	type II HBS	0.42 (IPEAT)	Yes
5	p.Arg79Cys	type II HBS	1.27 (IPEAT)	Yes
6	p.Arg79His	type II HBS	1.32 (IPEAT)	Yes
7	p.Leu131Phe	type II HBS	0.67 (GP-LGACN)	Yes
8	p.Val137Ala	type II PE	0.81 (GP-LGACN)	Yes
9	p.Phe155del	type I	-	Yes
10	p.Lys157Arg	type I	-	Yes
11	p.Arg177Cys	type II HBS	0.48 (LVSAN)	Yes
12	p.Gly199Arg	type II PE	0.98 (GP-SLTFN) 0.79 (LQPLD)	No
13	p.Asn224His	type II	0.71 (GP-WVSNK)	No
14	p.Asn224His	type II	0.69 (GP-WVSNK)	No
15	p.Asn224His	type II	0.77 (GP-WVSNK)	No
16	p.Glu227Lys	type II	1.28 (GP-WVSNK)	No
17	p.Glu227Lys	type II	1.44 (GP-WVSNK)	No
18	p.Glu227Lys	type II	1.32 (GP-WVSNK)	No
19	p.Asn240Lys	type II RS	-	Yes
20	p.Phe271Ser	type I	-	Yes
21	p.Lys273Glu	type II PE	0.79 (ELFYK)	No
22	p.Met283Val	type II RS/PE	-	Yes
23	p.Met283Ile	type II RS/PE	0.83 (ADGES)	Yes
24	p.Met283Lys	type II PE	-	Yes
25	IVS2-2 A>T & p.Ala416Ser	type I (type II RS)[d]	- -	- Yes
26	p.Ala416Ser	type II RS	-	Yes
27	p.Ala416Ser & p.Ser397Leu	(type II RS)[d] type I	- -	Yes -
28	p.Arg425del	type II RS	0.55 (SLNPN)	No
29	p.Arg425His	type II RS	0.58 (SLNPN)	No
30	p.Arg425Cys	type II RS	0.44 (SLNPN)	No
31	p.Ser426Leu	type II RS	0.43 (SLNPN)	Yes
32	p.Pro439Thr	type II PE	0.79 (ANRPF)	Yes
33	p.Leu441Pro	type I	-	Yes
34	p.Pro461Ser	type II PE	0.84 (VANPC)	Yes

HBS, heparin-binding site; PE, pleiotropic effect; RS, reactive site.

[a] Mutation as identified by genetic analysis.

[b] As detected by liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry analysis.

[c] Note that the p.Val30Glu mutation is expected to cause a transient quantitative type I deficiency, as described by Navarro-Fernandez et al. (2016) [35]. However, presence of variant proteoform suggests a qualitative type II deficiency. Therefore, subtype is marked as unknown.

[d] These samples contained double mutations of type I and type II RS, but are classified as type I due to the dominant effect of the type I mutation.

identified a total of 34 patients as having a qualitative ATD using the LC-MRM-MS test.

Variant proteoforms were observed in 4 mutations classified as type I ATD (p.Phe155del, p.Lys157Arg, p.Phe271Ser, p.Leu441Pro), which were expected to merely exert a quantitative effect and no expression or secretion of the mutant proteoform. Similarly, variant peptides were observed in samples that did not show a deviating qual/quant ratio. This was the case for the p.Ala416Ser mutation, which was located outside of the coverage of the wild-type peptides, but for which the mutation created a new tryptic variant peptide that could be observed. Two mutations at site Met-283 (p.Met283Lys and p.Met283Val) should theoretically have led to a deviating qual/quant ratio for the wild-type peptide ADGES. Variant peptides could be observed for both samples which also presented with low overall AT concentrations of 0.57 and 0.72 μmol/L (for p.Met283Lys and p.Met283Val, respectively), strengthening our initial hypothesis that the low overall AT concentration in these samples is caused by a low concentration of variant proteoform hampering the identification of the mutation through qual/quant ratios.

3.4 | Identification of abnormal glycosylation

Beyond *SERPINC1* mutations, ATD may be caused by abnormal glycosylation of AT, for instance as a result of CDGs. Whereas mutations are fixed variations, glycosylation is a variable process that may be influenced by CDG subtypes or temporal fluctuations. Thus, the impact of CDG on the glycopeptides was expected to show larger variability than that of mutations on regular peptides. To characterize the degree of hypoglycosylation of AT proteoforms in CDG patient plasma the 4 glycosylation sites of AT were monitored by measuring 4 glycopeptides, each targeting a single glycosylation site. One glycopeptide, GP-KANK, monitors the site (Asn-167) known to be hypoglycosylated naturally [36], leading to the distinction between 2 main proteoforms of AT; α-AT and β-AT, the latter contributing to ∼10% of the total AT in healthy adults [37]. Given the natural variability in this glycosylation site, it was anticipated that CDGs would have the largest effect on GP-KANK. Furthermore, a previous study showed that a second glycosylation site may be affected in CDG patients [30].

Significant differences were observed in the glycosylation of CDG patients vs controls for 2 glycopeptides (Figure 3A, B). Indeed, the largest difference was observed for glycopeptide KANK (GP-KANK), with a median normalized qual/quant ratio of 0.68 in CDG patients compared with 1 in controls ($P < .005$). In agreement with the study by de la Morena-Barrio et al. [30], a second significant change was found for GP-WVSNK, reflecting Asn-224, which showed a median normalized qual/quant ratio of 0.93 in CDG patients compared with 0.99 in controls ($P = .014$). Interestingly, 27 out of 41 (65.9%) CDG patients had a GP-KANK ratio below the lowest ratio found in healthy controls (0.83). As expected, a larger variation in glycosylation was observed as compared with mutations, with the qual/quant ratio for GP-KANK ranging between 0.42 and 1.07 in CDG patients. Samples with an aberrant glycopeptide/quant ratio were marked to likely harbor a glycosylation defect.

3.5 | Correlation between glycosylation and activity

Incorrect glycosylation of AT affects its functionality [30]. To investigate this, the correlation between the functionality of AT based on the diagnostic activity test was compared with the glycopeptide/quant ratios for CDG patients. Significant linear correlations with activity were found for both GP-KANK ($P < .005$) and GP-WVSNK ($P = .03$) ratios when assessed individually (Figure 3C, D), supporting a correlation between the degree of wild-type glycosylation and AT functionality. However, hypoglycosylation also affects the overall AT concentration due to lowered secretion. Thus, linear regression analysis of the overall AT concentration, GP-KANK concentration, and GP-KANK ratio vs the activity was performed, revealing GP-KANK to be the best predictor of activity (as reflected by higher r-squared values of 0.51 and 0.46 for GP-KANK concentration and ratio vs 0.42 for overall AT concentration). Combining these variables in a multiple linear regression mode revealed that the best model (based on the lowest Akaike Information Criterion) for predicting the activity was $Activity = -18.57 + 26.58 * quantifier + 78.32 * GP{-}KANK$ (see also Supplementary Table S1). Together, this indicates that the LC-MRM-MS results may be used to (partially) predict the functionality of AT.

3.6 | Identification of ATD patients by LC-MRM-MS vs activity test

In a diagnostic setting, the AT activity test serves as a first-line test to identify ATD in patients suspected of having thrombophilia. A cut-off of 80% activity is often applied (equal to the RI of the here applied activity test), classifying samples below 80% as ATD. The diagnostic sensitivity of the activity tests and the LC-MRM-MS test for the ATD samples studied here are shown in Table 3. Interestingly, both tests performed well for type I deficiencies, with 98.1% and 100% identified samples for the activity and LC-MRM-MS test, respectively. However, the activity test only identified 56.8% of the type II deficiencies, with the lowest rate found for type II pleiotropic effect deficiencies (36.4%), which is in line with previous studies reporting low sensitivity of activity tests for type II heparin-binding site (HBS) ATD [14,38]. In stark contrast, the LC-MRM-MS test identified 60.5% of the type II deficiencies based on AT quantity alone, rising to 100% when combining the quantitative and qualitative information. Of note, 8 of the 17 patients (47.0 %) missed by the activity test did suffer from a thrombotic event, indicating the clinical relevance of identifying these patients.

For CDG patients, screening for ATD (among other thrombophilias) is suggested to be performed at regular intervals or under provoking conditions [33,34]. It is not known nor expected that CDG patients are at constant risk for thrombophilia, as their coagulation parameters may fluctuate over time, depending on the extent to which the glycosylation

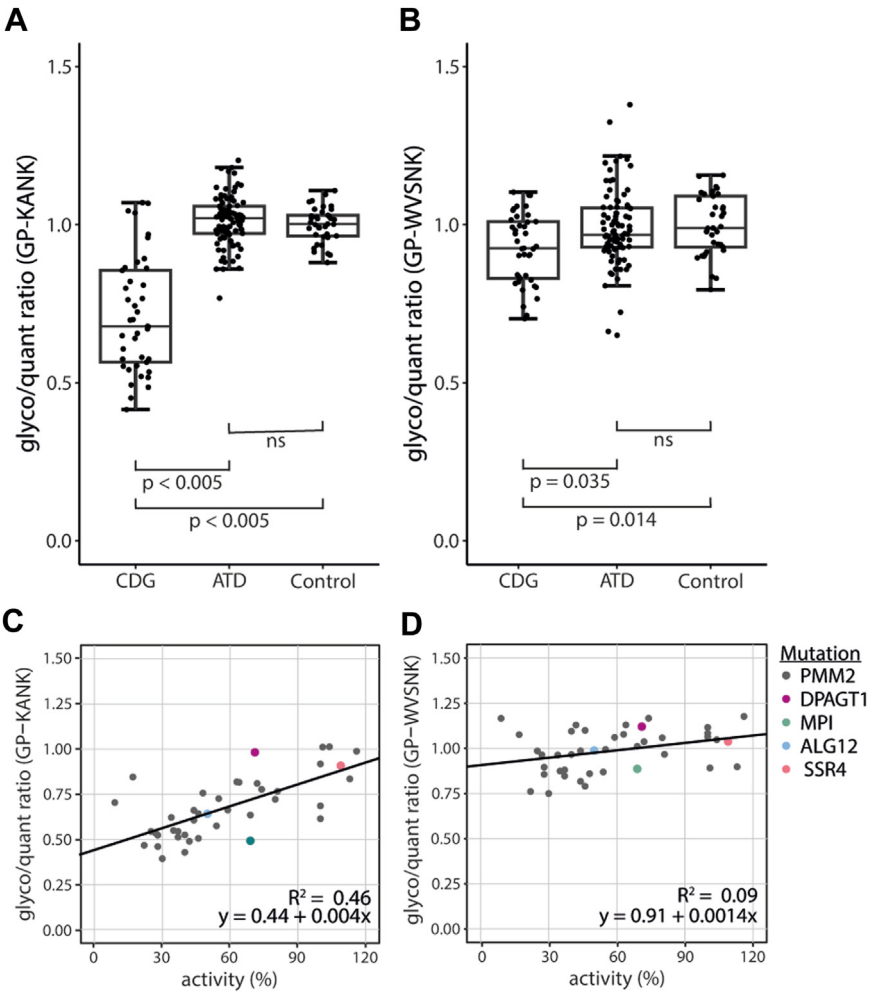


FIGURE 3 Analysis of glyco/quant ratio data for GP-KANK and GP-WVSNK. A-B) Boxplots for the normalized glyco/quant ratios for GP-KANK and GP-WVSNK, respectively. ns = not significant. **C-D)** Linear regression analysis for glyco/quant ratios vs the activity for GP-KANK (C) and GP-WVSNK (D), respectively.

deficiency may be remitted. Therefore, the LC-MRM-MS test was compared with the activity test (see also Supplementary Table S2) for identifying those at risk. The LC-MRM-MS test identified a slightly larger percentage of patients as ATD compared with the activity test (87.8% vs 75.6% for the LC-MRM-MS and activity test, respectively. Interestingly, the 5 patients not identified by the MS test also had normal values (ranging between 100% and 116%) in the activity test and only slightly increased aberrant transferrin values (CDG-marker, see Supplementary Table S3), suggesting that these patients were at low risk for thrombosis during sampling.

Overall, in this cohort of ATD and CDG patients, the LC-MRM-MS test provides high diagnostic sensitivity, with increased sensitivity in the case of ATD patients compared with the activity test, and comparable sensitivity compared with contemporary tests for identifying discrepancies in CDG patients.

4 | DISCUSSION

Diagnosing ATD by MS-based precision diagnostics is a valuable concept, but evidence for the clinical performance of this test was needed. There is a need to diagnose ATD more accurately in patients with ATD caused by *SERPINC1* defects, specifically in the ATD-type II HBS subtype. Moreover, the availability of a molecular diagnosis could enable studies toward targeted treatments. To address this need, AT was evaluated using our LC-MRM-MS test in a cohort of 91 patients with ATD caused by *SERPINC1* defects. By combining quantitative and qualitative data, the tests' diagnostic sensitivity for ATD outperformed that of the current activity test with an identification rate of 100% vs 81.1% for the activity test (Table 3). The test could also identify type II HBS mutations known to be difficult to diagnose through activity tests, such as p.Pro73Leu (AT Basel), p.Leu131Phe (AT Budapest III), and p.Arg79His (AT Padua). Compared with the activity test, the LC-MRM-MS test identified additional patients and enabled ATD subtyping, highlighting its potential to improve the current diagnostic pathway. Taken together, this study strongly supports the notion that molecular characterization of AT proteoforms improves diagnostic performance and enables identification of patients with *SERPINC1* defects in the diagnostic "grey zone" who are at risk for misdiagnosis.

AT carries 4 N-glycosylation sites, and altered glycosylation has been reported to influence AT activity. Consequently, glycosylation

TABLE 3 Diagnostic sensitivity of the activity test and the liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry test for ATD patients.

	Activity		LC-MRM-MS					
			Quantitative		Qualitative		Combined	
	N	%	N	%	N	%	N	%
ATD	73/90[a]	81.1	76/91	83.5	34/91	37.4	91/91	100
Type I	52/53	98.1	53/53	100	6/53[b]	11.3	43/43	100
Type II	21/37[a]	56.8	23/38	60.5	28/38	73.7	39/39	100
HBS	6/8	75.0	4/8	50.0	6/8	75.0	8/8	100
PE	4/11	36.4	10/11	90.9	7/11	63.6	11/11	100
RS	4/8[a]	50.0	2/9	22.2	8/9	88.9	9/9	100
NA	7/10	70.0	7/10	70.0	7/10	70.0	11/11	100

Identification by the activity test entailed an activity <80 %. For LC-MRM-MS identification, a quantitative defect entailed a concentration below the reference intervals (1.07 μmol/L). A qualitative defect entailed a deviating qual/quant ratio and/or a measured variant peptide.

ATD, antithrombin deficiency; CDG, congenital disorders of glycosylation; HBS, heparin-binding site; LC-MRM-MS, liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry analysis; PE, pleiotropic effect; RS, reactive site; NA, not available.

[a] Missing activity value for one sample.

[b] Two samples contained a combination of a type I and type II mutation and were classified as type I due to the dominant phenotype of this mutation.

was included in our LC-MRM-MS test. Patients with CDG carry altered glycosylation and can have constitutive or transient ATD. Current ATD activity tests do not target AT glycosylation specifically, while studies investigating ATD in CDG patients found low AT activity levels in approximately 80% of PMM2-CDG patients leading to a high occurrence of VTE (~10%) [33,39]. To assess the performance of our LC-MRM-MS test in the identification of ATD in CDG patients, AT was measured in a cohort of 41 patients with CDG. We identified a significant difference in the glycopeptide/quant ratios of patients compared with controls for GP-KANK and GP-WVSNK (representing glycosylation sites Asn-167 and Asn-224). Regarding the diagnostic sensitivity, the test classified a slightly higher percentage of patients in the ATD group compared with the activity test (87.8% vs 75.6%) indicating that the test performs similarly to the activity test with regards to identifying CDG patients at risk for thrombosis. As we currently do not have a "gold standard" for CDG patients, further evaluation in relation to VTE incidence would be warranted in the CDG population.

Beyond diagnostic performance, our findings open the discussion on the definition of type I and type II ATD. Classically, type I AD is characterized by low activity and low antigen levels, and type II ATD by low activity and normal to slightly lower antigen levels. In this study, 23 out of 38 (60.5%) type II ATD patients showed low quantities of AT, with many of the samples even showing similar concentrations to type I patients. Because type I ATD is typically regarded as more pathogenic than type II, these low quantities suggest that certain type II deficiencies should likely be regarded equally pathogenic as type I ATD. Conversely, variant peptides were detected in ATDs currently classified as type I, specifically p.Phe155del, p.Lys157Arg, p.Phe271Ser, and p.Leu441Pro. These results suggest that certain molecular variants must be reclassified and that the current classification system only provides a crude classification, likely not in line with the actual disease severity. Importantly, the clinical phenotype and severity of specific type II ATD at the molecular level have not systematically been studied. However, the presence of variants could lead to either ameliorating effects, such as retained activity in the β-AT proteoform of certain HBS mutations [40], or dominant negative effects [27,41]. Thus, proteoform analysis allows the establishment of a more refined classification system that may better resolve the categorization of patients at high and low risk for thrombosis based on molar quantitation combined with proteoform identification.

There is clear potential for this test to improve our understanding of ATD, but for it to provide added value in a diagnostic setting, it must improve the current clinical gap. For ATD, this entails the reduction of diagnostic uncertainty, especially for patients with borderline activity values and/or improved risk stratification of patients. For both instances, the test could be applied as an "add-on" test to the activity test to 1) verify the absence/presence of ATD in borderline cases, and 2) provide molecular information to better estimate thrombosis risk. Although the latter objective requires longitudinal studies investigating the thrombosis risk of specific AT variants, the feasibility of the first objective was partially verified in this study. Part (47.0%) of the additional ATD patients identified by LC-MRM-MS, but not by the activity test, already suffered from a clinical event, highlighting the inadequacy of the current test. Furthermore, this is a clear example of conventional "imprecision diagnostics" in our reactive healthcare system in which patients first have to undergo a clinical event, potentially causing lifelong damage, for a disease to be uncovered [42,43]. Current guidelines are hesitant toward thrombophilia screening, as current thrombophilia tests only identify a cause in less than half of the patients, and knowledge of the cause often does not alter the treatment strategy [11]. Thus, with the AT LC-MRM-MS test providing both a more refined approach to identifying ATD and offering molecular insight into the disease,

driving therapeutic developments, the thrombophilia community should be aware that an ounce of prevention by next-generation test screening can be worth a pound of cure.

The results warrant exploration of the full clinical performance in a longitudinal setup, for instance by comparing current practice (activity test) with the comparator test (LC-MRM-MS) in patients presenting with thrombophilia. As was the case for the current study, the low prevalence of ATD in the general population (1:400 to 1:600) may complicate the design of such a diagnostic accuracy study potentially requiring alternative approaches [26,44]. However, provided sufficient ATD cases are enrolled, and patient follow-up is sufficiently long, a well-designed diagnostic accuracy study may also allow for the development of risk stratification based on ATD molecular variants.

Although we believe that protein-level diagnostics offer valuable information that cannot be provided by functional or genetic testing, the LC-MRM-MS test is not omnipotent. The test performs well in identifying quantitative ATD but cannot discriminate between hereditary type I ATD and transient ATD. Consequently, the exclusion of transient factors, presence of family history, and/or genetic sequencing is still required to definitively classify a hereditary type I ATD, although these are also required when using activity tests. Furthermore, analogous to clinical care pathways, which must be improved continuously with novel insights and technologies, the LC-MRM-MS test also requires continuous development. In this study, the genetic and clinical information of the samples was essential to establish and verify a data analysis strategy and develop variant proteoform-specific transitions. The test now identifies many ATD mutations down to the amino acid levels, such as AT Basel, AT Padua I, and AT Budapest III, which occur in high frequencies in Europe [45,46]. Additionally, variant peptides for type II ATD mutations that were not present in the current cohort can be included for identification upon encounter. Of note, as the LC-MRM-MS technique is only established in specialized laboratories, centralized expert centers are envisioned to facilitate access to the in-house developed molecular AT-test, which only requires a small sample volume (<30 uL), provides stable results over multiple freeze/thaw cycles, and costs currently < $100 per test.

With the future vision of P5 medicine often focused on novel treatment strategies, such as gene therapy [47], we must not overlook that the clinical pathway is only as strong as each of its elements. For ATD, the current clinical pathway relies on a diagnostic strategy that may misdiagnose patients and create a clinically heterogeneous patient stratification. The LC-MRM-MS test refines the ATD diagnosis, enabling an improved diagnostic pathway and holds potential for improved patient stratification. To accomplish better patient management, tailored interventions targeting specific patient groups must be developed, for which molecular insight is essential, analogous to, for example, the oncology field where genotyping cancers allows personalized treatment [48]. Taken together, the LC-MRM-MS test enables the detection and quantification of harmful AT proteoforms which diminishes diagnostic uncertainty that hemostasis doctors face in the current ATD clinical care pathway, thereby bringing ATD "precision diagnostic and healthcare" into the 21st century.

ACKNOWLEDGMENTS

We thank all patients who contributed to this study. Furthermore, we thank ThermoFisher for providing the CaptureSelect α-Antithrombin used in the assay.

AUTHOR CONTRIBUTIONS

L.R.R. designed the study. J.C. and M.E.M.B. provided resources. M.K. performed the investigation and formal analysis. C.M.C. and L.R.R. reviewed the data. M.K. and L.R.R. wrote the manuscript. All authors reviewed and approved the final manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2024.10.005