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CASE REPORT

Unraveling a borderline antithrombin deficiency case with quantitative mass spectrometry

Mirjam Kruijt[1] | Liselotte M. van der Pol[2] | Jeroen Eikenboom[2] | Harjo J. Verburg[3] | Christa M. Cobbaert[1] | L. Renee Ruhaak[1]

[1]Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Center, Leiden, The Netherlands

[2]Department of Internal Medicine, Division of Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, The Netherlands

[3]Department of Gynecology and Fertility, Leiden University Medical Center, Leiden, The Netherlands

Correspondence
Mirjam Kruijt, Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Center, Office E2-27, Albinusdreef 2, 2333 ZA Leiden, the Netherlands.
Email: m.kruijt@lumc.nl

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Abstract

Antithrombin deficiency diagnostics by first-line activity tests suffer from a lack of sensitivity sometimes resulting in diagnostic uncertainty. We here present a case of a woman with recurrent pregnancy loss who was screened for inherited thrombophilia. Antithrombin activity was borderline low, resulting in uncertainty about the correct diagnosis. Using a mass spectrometry-based test, the antithrombin protein of the patient was characterized at the molecular level and a heterozygous p.Pro73Leu mutation was identified. The mutation, also known as antithrombin "Basel," increases the risk of venous thromboembolism and obstetric complications. This case is illustrative of current antithrombin deficiency screening, in which diagnoses may be missed by traditional diagnostics. Next-generation protein diagnostics by mass spectrometry provides molecular insight into the proteoforms present *in vivo*. This information is essential for laboratory specialists and clinicians to unambiguously diagnose patients and will aid in evolving healthcare from traditional to precision diagnostics.

KEYWORDS
antithrombin, antithrombin deficiency, mass spectrometry, molecular characterization, next-generation protein diagnostics, recurrent pregnancy loss

1 | INTRODUCTION

Antithrombin (AT) deficiency is a severe coagulation disorder with a 5- to 16-fold increased risk of venous thromboembolism (VTE).[1,2] Yet, diagnosis of AT deficiency is rather crude with current first-line activity tests measuring overall activity, potentially generating diagnostic uncertainty. Clinicians may therefore question the clinical relevance of results around the clinical decision levels (Figure 1A), even more so as the diagnosis may have an impact on clinical decisions and management.

In pregnant women, AT deficiency poses an additional risk for thrombosis as pregnancy by itself already increases the risk of VTE by 5- to 50-fold.[3] Furthermore, obstetric complications, such as recurrent pregnancy loss (RPL), are increased in women with AT deficiency.[4-6] Recommendations and guidelines for thrombophilia screening in pregnant women, similar to general AT deficiency guidelines, are often conflicting or report insufficient evidence.[7-9] These guidelines justify thrombophilia screening only in women presenting with RPL and a positive (family) history of VTE, thereby potentially missing AT deficiency

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diagnoses in women with unexplained RPL. The lack of evidence for a role of AT deficiency in RPL might originate from the heterogeneity of AT deficiencies, as more than 350 genetic AT variants have been reported, in addition to post-translationally introduced molecular variation. Each molecular AT form may potentially carry its own clinical phenotype. Current larger studies, upon which guidelines are based, often group all types of AT deficiency to generate sufficient power for statistical analysis, leading to underestimation of RPL risk of specific molecular AT forms. Smaller studies and case reports describing specific well-characterized AT deficiencies do show a link with obstetric complications.[10,11] We here present a case report that illustrates the need for molecularly defined AT deficiency screening at the protein level.

2 | CASE PRESENTATION

A 32-year-old woman presented at our obstetric clinic with RPL. Within a year, she had experienced three early miscarriages (<13 weeks of pregnancy). Karyotyping of both patient and partner were normal and pelvic ultrasound of the patient did not reveal abnormalities. Autoantibody testing was negative (lupus anticoagulant, anti-β2 glycoprotein antibodies, anti-cardiolipin antibodies, thyroid peroxidase antibodies) and thyroid-stimulating hormone level was normal. Both patient and partner are non-smokers, non-drug users, have a normal body mass index and have a low alcohol intake (<1 U/day). The patient did not have a positive family history of either miscarriage or VTE. To assess eligibility for participation in a study assessing the effect of low molecular weight heparin (LMWH) on pregnancy outcome

Essentials

- First-line antithrombin (AT) activity tests are known to lack sensitivity.
- A woman with recurrent pregnancy loss could not be clearly diagnosed as AT deficient by activity tests.
- Molecular characterization of AT by mass spectrometry provided an unambiguous diagnosis for the patient.
- Next-generation protein diagnostics by mass spectrometry provides valuable information for clinician and patient.

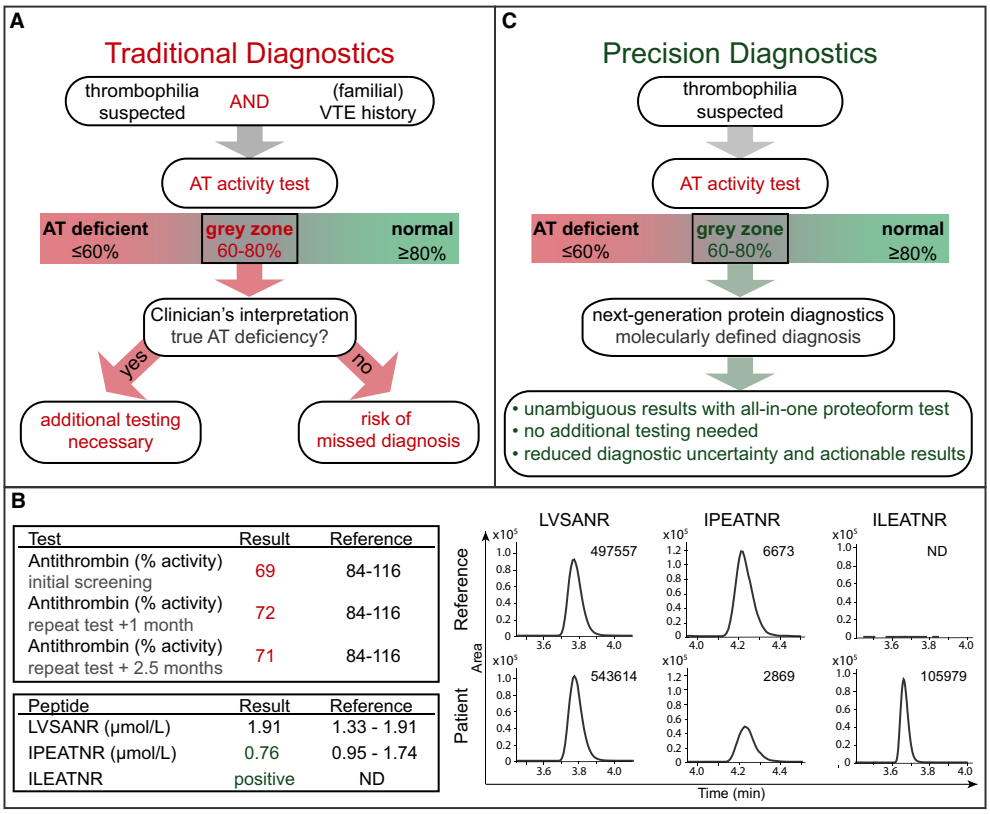

FIGURE 1 Current and proposed recommendation for antithrombin (AT) deficiency screening. A, Current strategy for patients suspected to have a thrombophilia. An AT activity test is performed but this test may generate ambiguous results. It is up to the clinician to decide whether results have clinical significance potentially leading to missed diagnoses. B, Results for AT activity tests (upper table) and liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry (LC-MRM-MS) test (lower table and graphs). Colored results in the tables indicate abnormalities. Low concentration for peptide IPEATNR in combination with the presence of mutated peptide ILEATNR ascertains a heterozygous p.Pro73Leu mutation. C, Proposed strategy for patients suspected to have a thrombophilia with ambiguous AT activity results. Using next-generation protein diagnostics, we provide a molecularly defined diagnosis to aid both clinician and patient in the diagnostic process for AT deficiency

in women with inherited thrombophilia, she was screened for inherited thrombophilia. The standard hereditary thrombophilia panel (deficiencies of protein C, protein S and AT, activated protein C resistance, factor V Leiden mutation, and factor II G20210A mutation) showed only mildly aberrant AT activity (Figure 1B). Initial AT activity was 69% and repeated measurements after 1 and 2.5 months indicated 72% and 71% activity, respectively (reference interval: 84%–116%; STA®-Stachrom® AT III). The patient was referred to the thrombosis and hemostasis out-patient clinic for counselling and determination of an indication for thrombosis prophylaxis. The responsible clinicians doubted whether the mildly lowered AT activity was of clinical significance and requested additional molecular characterization of AT to ensure correct diagnosis.

3 | MOLECULAR CHARACTERIZATION OF AT USING MASS SPECTROMETRY

In addition to the more than 350 reported possible mutations in AT, AT deficiency may be caused by an altered glycosylation status of the protein.[12] Together, this generates a large variety in potential molecular forms of AT, the so-called proteoforms. AT activity tests do not provide any molecular insight into the proteoforms underlying an AT activity result. Such insight is necessary in ambiguous cases that are currently inconclusive and therefore there is a clinical need for more informative and less ambiguous tests that may complement the current first-line activity tests.

To address this unmet clinical need, a mass spectrometry-based test was developed that concomitantly determines the concentration, frequent mutations, and glycosylation status of AT at the protein level.[13] The combination of these three features, obtained by directly analyzing the protein originating from the patient, is a unique ability of mass spectrometry, whereas the protein may only be measured indirectly by activity tests or genetic sequencing. In short, AT is purified from plasma and proteolytically digested into proteotypic peptides, which are quantified using liquid chromatography coupled to multiple-reaction-monitoring mass spectrometry (LC-MRM-MS). The signal of LVSANR, a non-mutation prone peptide, facilitates the quantitation of AT in μmol/L, while the monitoring of specific peptides can identify mutations or alterations in glycosylation. The LC-MRM-MS test provides molecular characterization of AT using only five μL of citrate plasma.

The LC-MRM-MS test was employed as an add-on test for the patient presenting with RPL. The results indicated a normal AT concentration level (LVSANR concentration 1.91 μmol/L; ref. 1.33–1.91 μmol/L) and a normal glycosylation status of the protein. However, the concentration of the peptide IPEATNR was only approximately half of the expected concentration (0.76 μmol/L; ref. 0.95–1.74 μmol/L; Figure 1B). A heterozygous mutation within this peptide was thus evident and additional analysis identified the mutated peptide ILEATNR, concordant with a p.Pro73Leu mutation, also known as the "Basel" mutation.[14] This proteoform has a heparin-binding site (HBS) defect reducing the AT activity and studies indicate high percentages of obstetric complications (34.7%–37.5%) in women with this mutation, matching the miscarriages of the patients.[10,11] The difficulty in diagnosing this mutation has been reported, for example by Orlando et al.,[15] who tested AT activity tests from four manufacturers on samples from seven patients with a known p.Pro73Leu mutation. Only one test identified all patients as deficient, whereas the other tests reported normal activities for almost all samples. Similar discrepancies arose for other HBS mutations as well indicating that HBS mutations may be missed using first-line activity tests.

Upon the diagnosis of a true AT deficiency, the patient described here has, per local protocol, an indication for LMWH as thrombosis prophylaxis postpartum to reduce VTE risk. The role of LMWH during pregnancy for improvement of pregnancy outcome is still debated.

4 | TOWARD PRECISION DIAGNOSTICS

Traditional AT activity tests have been and continue to be crude screening methods for detecting AT deficiency in the general population. However, these activity tests are "blind" for dysfunctional AT proteoforms. In our academic hospital, AT activities between 60% and 80% are regarded as ambiguous and additional information would aid clinicians to ensure correct diagnoses and fitting treatment.[12,16,17] In these cases next-generation protein diagnostics by mass spectrometry provides direct insight into the AT proteoforms present, thereby refining the diagnosis of AT deficiency (Figure 1C).

This case demonstrates a first proof-of-principle of molecular characterization of AT by mass spectrometry, where the technology was able to provide a direct and clear diagnosis, allowing the clinician to make an informed decision for a patient who would otherwise have fallen in a clinical gap. The strength of mass spectrometry lies in the ability to provide complete molecular characterization of the protein that is present in the patient *in vivo* in a single test, while a combination of activity and genetic tests can only provide the promise of a protein. Post-translational modifications, for which activity and genetic tests are blind, can also lead to AT deficiency,[18] further highlighting the importance of analyzing proteoforms. While the application of mass spectrometry for clinical diagnoses is in its infancy, the technology is increasingly available in clinical (chemistry) laboratories and we believe highlights of its applicability combined with increased experience in protein quantitation by mass spectrometry will facilitate a more general application of next-generation protein diagnostics by mass spectrometry—as add-on tests—as well as its extrapolation to other coagulation proteins.

Activity tests will remain the first-line test for AT deficiency screening, due to the wide availability on automated analyzers. In most cases, the activity results will enable a clear diagnosis of AT deficiency. However, for cases with ambiguous results, next-generation protein diagnostics by mass spectrometry may be employed to provide clarity for patients who can otherwise not be diagnosed. Insight into the specific proteoforms of AT and their clinical phenotype may give rise to more specific treatments, such as for RPL associated with this p.Pro73Leu mutation, or identify new risks associated only with specific mutations or glycosylation aberrances.

In conclusion, this case report highlights the inadequacies that clinicians and patients face using current AT activity tests and guidelines for detection of AT deficiency. We show the potential of our in-house–developed LC-MRM-MS test as an add-on for molecular characterization of AT, providing a molecularly defined AT deficiency diagnosis with an all-in-one test. The time for next-generation protein diagnostics to improve clinical care for patients with AT deficiency is now. We therefore call on laboratory specialists to realize the importance of precision diagnostics for evolving health care. Unraveling the molecular defects of AT will enable better understanding of this complex and heterogeneous disease and aid advancement of patient care.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

H.J. Verburg first saw the patient and referred her for further counselling. L.M. van der Pol and J. Eikenboom evaluated activity test results and consulted C.M. Cobbaert regarding the diagnostic uncertainty they experienced with the AT activity test results. The suggestion for additional testing was given by C.M. Cobbaert. M. Kruijt performed the LC-MRM-MS test. M. Kruijt and L.R. Ruhaak interpreted the data and wrote the manuscript. C.M. Cobbaert, L.M. van der Pol, J. Eikenboom, and H.J. Verburg critically reviewed the manuscript.

ORCID

Mirjam Kruijt https://orcid.org/0000-0002-4520-7385
Liselotte M. van der Pol https://orcid.org/0000-0002-8663-6418
Jeroen Eikenboom https://orcid.org/0000-0002-3268-5759
Harjo J. Verburg https://orcid.org/0000-0001-5213-3276
Christa M. Cobbaert https://orcid.org/0000-0003-3565-1404
L. Renee Ruhaak https://orcid.org/0000-0003-3737-3807

REFERENCES

1. Di Minno MND, Ambrosino P, Ageno W, Rosendaal F, Di Minno G, Dentali F. Natural anticoagulants deficiency and the risk of venous thromboembolism: a meta-analysis of observational studies. *Thromb Res*. 2015;135:923-932. doi:10.1016/j.thromres.2015.03.010
2. Middeldorp S. Is thrombophilia testing useful? *Hematology*. 2011;2011:150-155. doi:10.1182/asheducation-2011.1.150
3. Campello E, Spiezia L, Adamo A, Simioni P. Thrombophilia, risk factors and prevention. *Expert Rev Hematol*. 2019;12:147-158. doi:10.1080/17474086.2019.1583555
4. Abbattista M, Gianniello F, Novembrino C, et al. Risk of pregnancy-related venous thromboembolism and obstetrical complications in women with inherited type I antithrombin deficiency: a retrospective, single-centre, cohort study. *Lancet Haematol*. 2020;7:e320-e328. doi:10.1016/S2352-3026(20)30007-7
5. Kraft J, Sunder-Plassmann R, Mannhalter C, et al. Women with homozygous AT deficiency type II heparin-binding site (HBS) are at high risk of pregnancy loss and pregnancy complications. *Ann Hematol*. 2017;96:1023-1031. doi:10.1007/s00277-017-2965-2
6. Ilonczai P, Oláh Z, Selmeczi A, et al. Management and outcome of pregnancies in women with antithrombin deficiency: a single-center experience and review of literature. *Blood Coagul Fibrinolysis*. 2015;26:798-804. doi:10.1097/mbc.0000000000000348
7. Bates SM, Middeldorp S, Rodger M, James AH, Greer I. Guidance for the treatment and prevention of obstetric-associated venous thromboembolism. *J Thromb Thrombolysis*. 2016;41:92-128. doi:10.1007/s11239-015-1309-0
8. Wu O, Robertson L, Twaddle S, et al. Screening for thrombophilia in high-risk situations: systematic review and cost-effectiveness analysis. The Thrombosis: Risk and Economic Assessment of Thrombophilia Screening (TREATS) study. *Health Technol Assess*. 2006;10:1-110. doi:10.3310/hta10110
9. Van Cott EM, Orlando C, Moore GW, Cooper PC, Meijer P, Marlar R. Recommendations for clinical laboratory testing for antithrombin deficiency; Communication from the SSC of the ISTH. *J Thromb Haemost*. 2020;18:17-22. doi:10.1111/jth.14648
10. Puurunen M, Salo P, Engelbarth S, Javela K, Perola M. Type II antithrombin deficiency caused by a founder mutation Pro73Leu in the Finnish population: clinical picture. *J Thromb Haemost*. 2013;11:1844-1849. doi:10.1111/jth.12364
11. Gindele R, Pénzes-Daku K, Balogh G, et al. Investigation of the differences in antithrombin to heparin binding among antithrombin budapest 3, basel, and padua mutations by biochemical and in silico methods. *Biomolecules*. 2021;11:544. doi:10.3390/biom11040544
12. Corral J, de la Morena-Barrio ME, Vicente V. The genetics of antithrombin. *Thromb Res*. 2018;169:23-29. doi:10.1016/j.thromres.2018.07.008
13. Ruhaak LR, Romijn FPHTM, Smit NPM, et al. Detecting molecular forms of antithrombin by LC-MRM-MS: defining the measurands. *Clin Chem Lab Med*. 2018;56:1704-1714. doi:10.1515/cclm-2017-1111
14. Chang JY, Tran TH. Antithrombin III Basel. Identification of a Pro-Leu substitution in a hereditary abnormal antithrombin with impaired heparin cofactor activity. *J Biol Chem*. 1986;261:1174-1176. doi:10.1016/S0021-9258(17)36071-4
15. Orlando C, Heylen O, Lissens W, Jochmans K. Antithrombin heparin binding site deficiency: a challenging diagnosis of a not so benign thrombophilia. *Thromb Res*. 2015;135:1179-1185. doi:10.1016/j.thromres.2015.03.013
16. James AH, Bates SM, Bauer KA, et al. Management of hereditary antithrombin deficiency in pregnancy. *Thromb Res*. 2017;157:41-45. doi:10.1016/j.thromres.2017.05.017
17. Pabinger I, Thaler J. How I treat patients with hereditary antithrombin deficiency. *Blood*. 2019;134:2346-2353. doi:10.1182/blood.2019002927
18. de la Morena-Barrio ME, Martínez-Martínez I, de Cos C, et al. Hypoglycosylation is a common finding in antithrombin deficiency in the absence of a SERPINC1 gene defect. *J Thromb Haemost*. 2016;14:1549-1560. doi:10.1111/jth.13372

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