Laboratory Investigations of Thrombophilia

Clinical and Practical Aspects

Akbar Dorgalaleh
Anvarjon Samadov
Editors

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1 An Overview of the Laboratory Diagnosis of Thrombophilia

Najmeh Lashgari and Ali Noroozi-Aghideh

1.1 Introduction

Thrombophilia is the predisposition to the development of abnormal blood clots caused by genetic or acquired risk factors that affect blood coagulation or vascular systems. The laboratory has a crucial role in the proper diagnosis and timely management of thrombophilia [1]. Therefore, this chapter aims to provide a brief overview of the laboratory tests used to diagnose thrombophilia, highlighting recent advancements in diagnostic methods and clinical applications.

Thrombophilia can be classified into inherited and acquired types, each involving abnormalities in coagulation factors, platelet function, or other components of the thrombosis and hemostasis or cardiovascular system. Inherited thrombophilias are usually due to mutations in genes encoding coagulation factors or platelet surface proteins, while acquired forms arise due to environmental conditions such as pregnancy, surgery, or antiphospholipid syndrome (APS) [1, 2].

Inherited thrombophilia: Inherited thrombophilia commonly includes the following genetic mutations:

- **Factor V Leiden**: A variant that makes factor V resistant to inactivation by activated protein C (APC), increasing the risk of thrombosis [1, 2].
- **Prothrombin G20210A**: Another variant in the *F2* gene, leading to higher plasma levels of prothrombin and increased risk of thrombosis [3].

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Table 1.1 Common laboratory tests for thrombophilia diagnosis

Test	Purpose	Common method
Factor V Leiden	Identifies mutation in Factor V gene	PCR, sequencing
Prothrombin G20210A	Detects prothrombin mutation	PCR
Protein C	Measures level of protein C	Functional assay, ELISA
Protein S	Measures level of protein S	Functional assay, ELISA
Antithrombin III	Measures level of antithrombin III	Functional assay

Natural anticoagulant deficiencies: The natural anticoagulants are protein C, protein S, and antithrombin III, which play central roles in regulating the coagulation process [4].

Acquired thrombophilia: Acquired conditions, such as antiphospholipid syndrome, pregnancy, and surgical procedures, also significantly increase the risk of thrombosis. The presence of antiphospholipid antibodies can interfere with the normal anticoagulant system, increasing the risk of thrombosis [5].

1.2 Laboratory Diagnosis of Thrombophilia

The diagnosis of thrombophilia requires a comprehensive approach involving clinical assessment, family history, and laboratory testing. Laboratory testing is crucial for confirming genetic mutations or coagulation factor deficiencies, which can guide treatment decisions (Table 1.1).

1.3 Coagulation Tests

Several coagulation tests can be used to detect abnormal clotting tendencies. The most common coagulation tests include:

Activated partial thromboplastin time (APTT) and **prothrombin time (PT)**: These tests are the most commonly used coagulation tests that measure the time it takes for blood to clot and may be shortened in patients with a thrombotic tendency, most notably increased factor VIII levels [1–4].

Thrombophilia screening includes a combination of tests assessing factors such as activated protein C resistance, protein S, protein C, and antithrombin levels, as well as Factor V Leiden and prothrombin G20210A variants [6].

Genetic testing for thrombophilia: Molecular testing is essential for diagnosing inherited thrombophilias, particularly Factor V Leiden and Prothrombin G20210A variants. Molecular methods like polymerase chain reaction (PCR), Sanger sequencing, or next-generation sequencing (NGS) can be used to identify these variants in patients at risk for thrombosis [7].

Laboratory diagnosis of antiphospholipid syndrome: The laboratory diagnosis of APS is mainly based on the detection of antiphospholipid (aPL) antibodies. The most common aPLs are listed below:

Lupus anticoagulant (LA): This is detected using tests like the dilute Russell viper venom time (dRVVT) and APTT [8].

Anticardiolipin (aCL) antibodies: Detected using enzyme-linked immunosorbent assay (ELISA) [8, 9].

Anti-β2-glycoprotein I antibodies: Also detected by ELISA [8–10].

APS may increase the risk of recurrent thrombosis and pregnancy loss [8–10].

1.4 Advances in the Diagnosis of Thrombophilia

Over the last decade, there have been significant advancements in the laboratory diagnosis of thrombophilia. These include the development of high-throughput genetic testing, improved clotting assays, and more reliable markers for detecting acquired thrombophilias.

1.5 High-Throughput Genetic Testing

Next-generation sequencing (NGS) is a new emerging technology that has made it easier to simultaneously screen for multiple thrombophilia-related genetic variants. This technique offers higher sensitivity and can be used for both known and novel genetic variants [9].

1.6 Biomarkers for Antiphospholipid Syndrome

New biomarkers are being explored for their diagnostic implications in APS, such as anti-β2-glycoprotein I antibodies, which may offer a more reliable diagnosis of APS in patients with a history of thrombosis [9, 10].

1.7 Screening for Multiple Thrombophilias

Several commercial panels are now available for the screening of multiple acquired and inherited thrombophilias, significantly improving the diagnostic efficiency [9, 10].

1.8 Challenges in Laboratory Diagnosis

Despite all these advances, there remain significant challenges in diagnosing thrombophilia, including:

Timing of testing: Thrombophilia testing, particularly clot-based assays, should ideally be performed during a period when the patient is not experiencing acute thrombosis or is off anticoagulant therapy to obtain reliable results [7, 8].

False positives/negatives: The presence of certain conditions, such as lupus or pregnancy, can influence test results, giving false-positive/negative results [7].

Interpretation of results: Genetic variants and coagulation tests require careful interpretation, as mutations in factor V and prothrombin do not always result in thrombosis, and deficiencies of natural anticoagulants may be compensated by other mechanisms in the body. Therefore, appropriate interpretation of these results by an expert hematologist may be mandatory [7, 8].

1.9 Clinical Implications

The results of thrombophilia tests are important for guiding clinical decisions, such as anticoagulation therapy, in patients with venous thromboembolism (VTE) or those at high risk. Genetic counseling is also important for patients and their families, as the genetic component of thrombophilia may affect multiple members of a family [9, 10].

1.10 Conclusion

The laboratory diagnosis of thrombophilia has evolved significantly with advancements in genetic testing and the identification of new biomarkers in the last decade. Despite all the challenges, an accurate diagnosis of thrombophilia is essential for the appropriate management of the patients and to reduce the risk of thrombosis.

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2 Laboratory Diagnosis of Congenital Antithrombin Deficiency

Mahmood Shams

2.1 Introduction

Antithrombin (AT) is a slow-acting serine protease inhibitor (serpin) that primarily deactivates thrombin (activated factor II [FIIa]) and FXa, with lesser effects on FIXa, FXIa, FXIIa, FVIIa, tissue plasminogen activator (tPA), urokinase, trypsin, plasmin, kallikrein, and complement enzyme C1 [1–3]. In addition, AT has a significant anti-inflammatory role through two main mechanisms: 1. it inhibits thrombin and FXa, which reduces the thrombin/FXa-mediated release of pro-inflammatory cytokines, such as interleukin (IL)-6 and IL-8, and, 2. it binds to endothelial glycosaminoglycans, such as heparin sulfate, promoting the production of the anti-inflammatory cytokine prostacyclin. With a half-life of approximately 2.4 days, AT is predominantly synthesized in the liver and circulates in a form with low inhibitory activity. However, the presence of heparin and other heparin-like glycosaminoglycans can enhance AT's inhibitory activity by up to a 1000-fold [1, 3, 4]. Hereditary AT deficiency was first described by Olav Egeberg in a Scandinavian family member with venous thromboembolism (VTE) [5].

Congenital AT deficiency is a relatively uncommon autosomal dominant disorder with a prevalence ranging from 1 in 500 individuals to 1 in 5000 individuals. In contrast, its prevalence among patients with VTE is estimated to be between 1 in 20 and 1 in 200 [1, 6–8]. This seemingly illogical prevalence distribution can be attributed to several factors:

1. Healthy individuals may present with falsely low AT activity due to the lack of repeated testing, as most cases of low AT levels yield normal values upon retesting, which is often attributable to laboratory errors.

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2. Near the normal antigen assay results in type II deficiencies, particularly type II-HBS (heparin-binding site), which may lead to false-negative diagnoses if activity assays are not performed.

AT deficiency is a significant risk factor for VTE and pregnancy loss [1, 9].

2.2 Classification of Antithrombin Deficiency

AT deficiency is classified into two types: type I (quantitative defect), characterized by proportionally reduced antigenic and activity levels, and type II (qualitative defect), marked by normal antigen levels but decreased activity. Type II is further subclassified into three types: type IIa (reactive site [II-RS]), type IIb (II-HBS), and type IIc (pleiotropic [II-PL]). Type II-RS is less common but more thrombogenic due to mutations at the thrombin-binding site. Type II-HBS deficiency arises from defects in the heparin-binding region of AT and is more prevalent but less thrombogenic. Type II-PL deficiency involves pleiotropic mutations near the reactive loop site [1, 10].

2.3 Laboratory Approach

Functional assays of AT are recommended as the first-line screening method for diagnosing AT deficiency. Patients with a strong family history of thrombosis, as well as young patients with thrombosis and no apparent family history, should be investigated for AT deficiency [11, 12].

Given the wide normal range of AT levels, it is essential to repeat low AT activity results with a new sample, as normal results are often obtained upon retesting. Furthermore, it is crucial to rule out acquired thrombophilic causes. Further evaluations, such as antigenic assays and molecular genetic testing, are often necessary for subclassifying AT deficiency. Although these tests primarily serve non-medical purposes, they are not universally accepted as a standard approach (Fig. 2.1) [1, 13, 14].

2.3.1 Functional Assay

The functional assay of AT is based on its ability to inhibit FIIa or FXa in the presence of heparin (heparin cofactor assay) or its absence (progressive activity assay). The latter is influenced by other serine proteases in the plasma, such as α2-macroglobulin. Notably, in the absence of heparin, the inhibitory rate of AT decreases significantly, resulting in lower sensitivity compared to the heparin cofactor assay. Consequently, the progressive activity assay is typically used as a corroborative test [15].

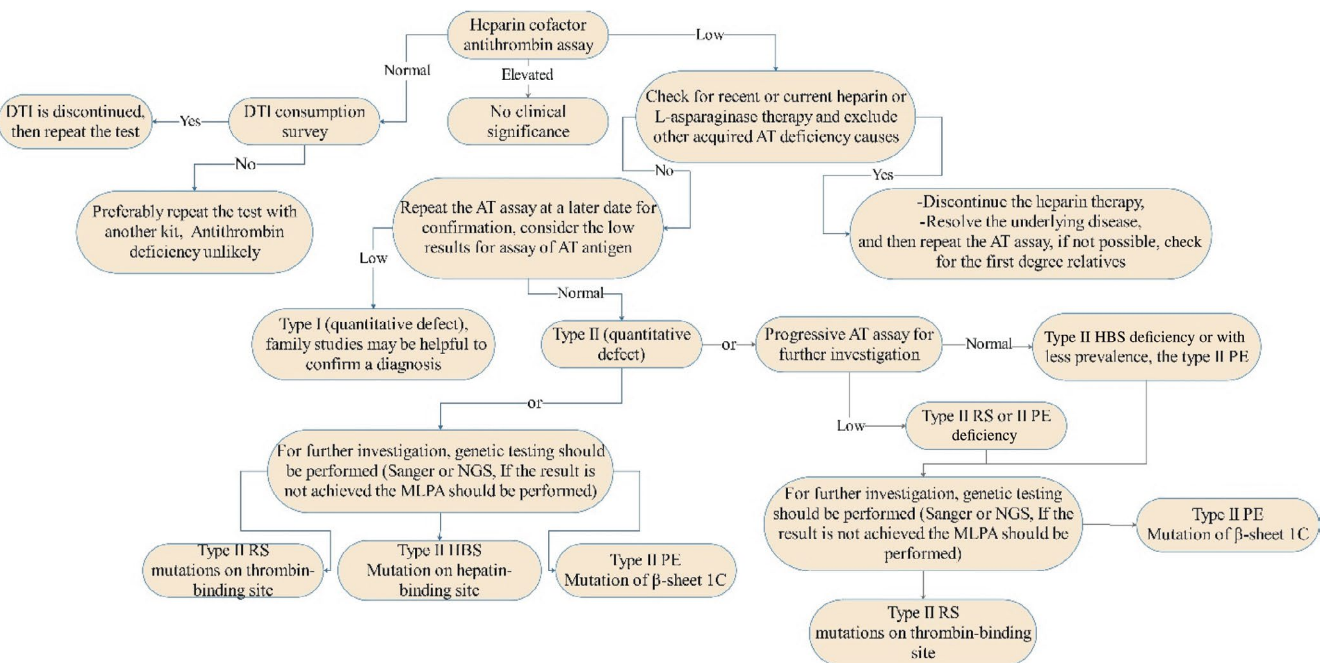

Fig. 2.1 Diagnostic algorithm for hereditary antithrombin (AT) deficiency. The functional assay serves as the cornerstone in diagnosing AT deficiency. In this context, the acquired causes of AT deficiency should be excluded. The antigenic assay may be considered, especially in cases with normal protein C and protein S levels. Molecular investigations in patients or first-degree relatives, along with antigen assays, are performed for further characterization or confirmation. *RS* reactive site, *HBS* heparin-binding site, *PL* pleiotropic, *NGS* next-generation sequencing, *MLPA* multiplex ligation-dependent probe amplification, *DTI* direct thrombin inhibitors

The functional assay can be performed using either clotting-based or chromogenic-based methods. However, due to its precision, the chromogenic assay is currently the preferred method for assessing AT activity. Various commercial kits employing different methodologies are available for detecting the antigenic and activity levels of AT, and we provide a general overview of these methods

2.3.1.1 Coagulometric Heparin Cofactor Assay

This assay is based on the ability of AT to prolong the clotting time in the presence of thrombin or FXa. Currently, due to their time-consuming nature and lower accuracy, the use of these methods is not common.

Principle of the Coagulometric Heparin Cofactor Assay

After preparing a defibrinated plasma sample using prolonged heating, the plasma is incubated with thrombin or, in some assays, FXa in excess. Adding heparin as an AT cofactor could accelerate the inhibitory reaction of AT. Finally, the fibrinogen solution is added to the mixture, and clotting time is measured, which is proportional to the AT activity.

2.3.1.2 Chromogenic Heparin Cofactor Assay and Progressive Activity Assay

Currently, most diagnostic methods for AT deficiency are predominantly based on amidolytic procedures. In these methods, as with the coagulometric techniques, heparin enhances the reaction rate of AT. These assays are generally more specific and sensitive than coagulometric assays, effectively eliminating the influence of other plasma inhibitors and thereby making the method more practical. The activity is typically assessed by the inhibition of bovine FIIa or FXa, human FXa, or FIIa in excess, all in the presence of heparin [11, 16, 17].

Principle of Chromogenic Heparin Cofactor Assay

Citrated plasma diluted in a buffer containing heparin or heparin sulfate is incubated with excess human or bovine FIIa or FXa. In some methods, the target factor (e.g., FXa or FIIa) should already be reconstituted in a buffer containing heparin or heparin sulfate. After adding the chromogenic substrate, the residual FXa (or FIIa) in the mixture hydrolyzes the chromogenic substrate (which mimics natural substrates), releasing *para*-nitroaniline (*p*-NA) as a chromophore. The reaction can be considered either a kinetic method or an endpoint method, with spectrophotometric measurements taken at 405 nm. In the former case, the reaction is monitored over a relevant time period using a kinetic reading protocol. In the endpoint method, the reaction is stopped with acid, and the absorbance is measured against water. In this context, a standard curve should be plotted on linear graph paper based on absorbance per minute (ΔA/min) for kinetic mode and standard absorbance against the AT activity in endpoint methods. The change in absorbance is inversely proportional to the AT activity.

Principle of the Chromogenic Progressive Activity Assay

This method is performed similarly to the heparin cofactor assay, with some differences, including the replacement of heparin in the dilution buffer with another component, such as polybrene, heparin neutralization, and an extended incubation time. Increasing the plasma volume during dilution preparation and extending the incubation time may enhance the sensitivity of the test.

Reference range: The normal reference range for plasma AT is 112–140 μg/mL. However, due to the presence of interlaboratory result variations, most laboratories report antigen and activity levels as percentages. In this regard, the reference range for adults and infants over 6 months of age is 80–120%. The normal range for heterozygote patients is around 40–60%, while full-term newborn infants have a lower reference range of about 39–87%. This range gradually increases to adult levels by 6 months of age. Women taking combined oral contraceptive pills and elderly men have slightly lower AT levels, which are considered physiological deficiencies. Premenopausal women typically have lower AT levels compared to postmenopausal women or men of the same age [1, 18].

Interfering variables: Direct FXa inhibitors such as rivaroxaban (Xarelto), apixaban (Eliquis), and edoxaban (Savaysa), as well as direct thrombin inhibitors like dabigatran (Pradaxa), efegatran, and inogatran, along with vitamin K antagonists, may falsely elevate AT activity, depending on the substrate used, which can mask AT deficiency. In such cases, discontinuation of the medication is recommended before retesting. The presence of inhibitors, particularly heparin cofactor II (HCII), a relatively specific thrombin inhibitor, can lead to an overestimation of AT plasma levels. Consequently, using FXa instead of FIIa can yield more reliable results for identifying AT deficiency, as it minimizes interference from HCII, α2-macroglobulin, α1-antitrypsin, and direct thrombin inhibitors [3, 14, 16, 19, 20]. Bovine FIIa reacts poorly with HCII, making human FIIa preferable in heparin cofactor assay methods [19]. Heparin therapy and acute thrombosis may transiently lead to a low level of AT [1]. Several studies have shown significant inter-assay variability, particularly in type II AT deficiency when different functional assay methods are employed [21]. To avoid diagnostic pitfalls in AT deficiency, it is crucial to exclude acquired causes and consider conditions such as clotting in the specimen, acute thrombosis, and preeclampsia, which can reduce AT levels [22, 23].

Interpretation: The functional AT assay is the primary method for diagnosing AT deficiency. Although both the heparin cofactor assay and the progressive AT assay can be used, the heparin cofactor assay may be more reliable due to the increased activity of AT in the presence of heparin. Nonetheless, these methods, along with antigen assays, can help differentiate various subtypes of AT deficiency (Table 2.1) [1, 24]. In cases of type II-HBS deficiency, both functional assays can assist in distinguishing this subtype from others, especially when anti-FXa is employed for diagnosis [25]. Type II-HBS can also be diagnosed using two-dimensional counter/crossed immunoelectrophoresis and mutation analysis [1]. It's important to note that there can be significant sensitivity differences

Table 2.1 Laboratory characterization of antithrombin deficiency

Type of defect	Heparin cofactor activity[a]	Progressive AT assay[b]	Antigen assay
Type I-Quantitative	Low	Low	Low
Type II-RS	Low	Low	Often normal
Type II-HBS	Low	Normal	Often normal
Type II-PE	Low	Varied	Normal or subnormal

RS reactive site, *HBS* heparin-binding site, *PE* pleiotropic, *AT* antithrombin
[a] AT activity is measured in the presence of heparin
[b] AT activity is measured in the absence or low concentration of heparin

between anti-FIIa- and anti-FXa-based assays in some cases of type II-RS deficiency. For instance, the AT Denver variant (p.426Ser > Leu) yields normal results with the anti-FXa-based assay but shows low levels with the bovine anti-FIIa-based assay. Interestingly, the AT Cambridge II variant (p.416Ala > Ser) can be detected by the heparin cofactor assay but not by the progressive AT assay [23]. Furthermore, the normal results of AT in the heterozygous form of type II-HBS are observed using an anti-FIIa-based assay. Therefore, anti-FXa-based assays are preferred over anti-FIIa-based assays [25, 26].

These contradictory results may be attributed to variations in the concentration or type of heparin used, incubation time, dilution buffer composition, sample dilution volume, and the target protease [13].

2.3.2 Immunological Assay of Antithrombin

An immunological assay of AT is necessary for the differentiation of type I AT deficiency from type II. However, it has been determined that subtype IIb has a much lower risk for thrombosis; therefore, the assay of the antigenic level may be clinically relevant [1, 27]. Different methods, such as latex immunoassay and ELISA (Enzyme-Linked Immunosorbent Assay) with different methodologies, are nowadays available for the assessment of AT concentration.

2.3.2.1 Microlatex Particle-Mediated Immunoassay

This assay is based on measuring the change in the turbidity of a microparticle suspension using a photometer.

Principle of the Antigenic Assay of Antithrombin Via Microlatex Particle-Mediated Immunoassay

In this method, diluted patient plasma, controls, and serial dilutions of a standard are added to disposable spectrophotometer cuvettes. Following this, microlatex particles containing antibodies specific to AT are added, and the mixture is agitated while starting a stopwatch. During incubation, the antigen-antibody reaction causes microlatex particles to aggregate, leading to a change in the optical absorption that

can be measured spectrophotometrically. A standard curve is created by plotting the relevant absorbance on the *y*-axis against the AT levels of each calibrator on the *x*-axis. The increase in absorption is directly proportional to the antigenic level of the sample.

Interfering variable*:* Samples with gross hemolysis, lipemia, or icterus should be rejected. To ensure accurate results, double centrifugation is necessary: the first centrifugation separates the plasma, while the second removes the platelets. Contamination with platelets can lead to spurious results; generally, platelet counts should be less than 10,000/μL in coagulation studies. Additionally, heparin therapy may transiently decrease AT levels. A high titer of the rheumatoid factor may also lead to an overestimation of the AT concentration.

2.3.2.2 ELISA Assay of Antithrombin

The ELISA method is a widely used technique for measuring the antigenic levels of AT.

Principle of Antigenic Assay of Antithrombin Using the ELISA Method

In this assay, diluted patient plasma, controls, and a normal pool plasma standard are added to a microplate pre-coated with a specific polyclonal antibody, followed by incubation. Unbound components are washed away, and a primary antibody is added, followed by further incubation. After washing the excess antibody, a conjugated secondary antibody is added to each well and incubated again. A chromogenic substrate is then added, followed by incubation, and a stop solution is introduced to each well. The absorbance is spectrophotometrically measured at 450 nm. The standard curve is generated, allowing for the determination of the antigen levels of AT. The absorption is directly proportional to the amount of AT antigen captured on the plate.

Reference range: Each laboratory should establish its own reference range, but it is generally considered to be between 80% and 120%. Pregnancy is associated with decreased concentrations of AT. A reduced AT concentration is also observed in neonates until about 6 months of age.

2.4 Molecular Diagnosis

The AT gene (*SERPINC1*) is located on chromosome 1q23-q25 and spans over 13 kb, containing 7 exons and 6 introns, coding for 432 amino acids [28]. There are 9 full and 1 partial Alu repeat elements located in introns 1, 2, 3B, 4, and 5, as well as a highly polymorphic trinucleotide repeat sequence in intron 4, which can be used for studying recurrent mutations and conducting linkage analysis in families with thrombosis [25]. The disorder is primarily inherited in an autosomal dominant manner, although type II-HBS often exhibits incomplete penetrance or an autosomal recessive pattern [24]. Most patients are heterozygous for the defect, displaying

AT activity values around 50%. The homozygous form is generally incompatible with life, frequently resulting in death in utero. However, type II-HBS can occur in a homozygous form and is associated with a high risk of VTE [25]. Information on mutations can be found in the human gene mutation database (HGMD) at http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1.

Many gene variations in the AT gene, including missense and nonsense mutations, small deletions, insertions, and splice-site mutations, have been reported so far. Interestingly, the AT gene suffers a low rate of common polymorphism, especially those with missense changes, revealing the gene's resistance to even minor genetic defects [13]. Most mutations causing type I deficiency are point mutations or insertion/deletion mutations, with fewer cases involving partial or complete gene segment deletions. Given the small size of the gene and high mutation rate, sequencing of the seven exons and flanking regions of *SERPINC1* is recommended for mutation identification via Sanger sequencing or next-generation sequencing (NGS). Additionally, methods such as multiplex ligation-dependent probe amplification (MLPA) and PCR/HPLC (polymerase chain reaction/denatured high-performance liquid chromatography) can be utilized for detecting large gene deletions [29, 30]. While molecular genetic testing is not routinely employed for diagnosing AT deficiency, several considerations are important:

1. The presence of heterogeneity in clinical presentation and varying risks of thrombosis among patients with similar anti-FXa activity assay results [13].
2. The current functional methods may fail to detect some cases with specific pathogenic mutations, such as the Cambridge II variant (c.1246G > T) [31].

In such instances, molecular characterization of the underlying mutation may be essential for accurate diagnosis and appropriate treatment [13]. Prenatal diagnosis may be considered in rare cases where a risk of homozygous or compound heterozygous AT deficiency is anticipated [1].

A new study by Morena-Barrio highlights the limitations of conventional testing methods for AT deficiency. In this setting, they discovered two novel *SERPINC1* variants, p.Glu227Lys and p.Asn224His, which affect N-glycosylation and, importantly, cause severe thrombophilia. Surprisingly, these variants cannot be detected through standard assays, suggesting the need to promote new or at least modify the functional assays and also the importance of genetic testing for accurate diagnosis [32].

2.5 Conclusion

AT deficiency is a significant risk factor for VTE, presenting a critical challenge in the management of affected patients. The elevated annual incidence of VTE in individuals with AT deficiency, coupled with a notably high rate of recurrence, highlights the specific need for accurate and timely diagnosis [17]. This is particularly important in the presence of additional underlying thrombotic risk factors, such as

obesity, pregnancy, and prolonged immobility, which can further exacerbate the risk of thrombosis. Awareness of AT deficiency is essential for healthcare providers, as early identification can lead to proactive management strategies that significantly reduce the likelihood of thrombotic events.

In clinical practice, functional assays of AT are typically employed as the first-line screening method for diagnosing AT deficiency. These assays measure the functional ability of AT to inhibit thrombin and FXa, providing an initial assessment of the patient's anticoagulant status. Following a positive screening result, immunological assays are conducted to distinguish between type I and II. Nonetheless, a report about the new variant that is undetectable with these routine workups emphasizes the continuous improvement of functional assays besides molecular analysis. In this context, to conclusively confirm the diagnosis, molecular investigations are used to identify specific genetic mutations associated with AT deficiency. The combination of these diagnostic approaches not only facilitates a comprehensive understanding of the patient's condition but also informs appropriate therapeutic interventions.

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Conflict of Interest None to declare.

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