On the diversity of antithrombin proteoforms: the role of a diagnostic mass spectrometry-based test for antithrombin deficiency

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General Introduction

based on
Antithrombin: Deficiency, Diversity, and
the future of Diagnostics

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Abstract

Our current healthcare system has its focus on reactive sick-care treating patients after symptoms have appeared and by applying generic and often suboptimal treatments. This creates high costs and pressure on our healthcare system which is not sustainable. Alternatively, P5 healthcare is proposed which focuses on five key elements: prevention, personalization, prediction, participation, psychocognition. To facilitate P5 healthcare, changes must be made in current clinical care pathways, for which antithrombin deficiency is a prime example. Hereditary antithrombin deficiency (ATD) is a genetic disorder, for which screening is instigated after a patient (or relative) has suffered from a thrombotic episode. Current diagnostic tests for ATD are lacking sensitivity and refinement to correctly classify patients. Lastly, treatments are generic and not based on the molecular defect underlying the ATD. To evolve the clinical pathway for ATD a better molecular understanding of (the different subtypes of) ATD is essential, and a molecular diagnostic test that analyzes all clinically relevant features of antithrombin is required. This review aims to compile information regarding clinically relevant molecular characteristics of antithrombin, the diversity of antithrombin (deficiency) in heath and disease, and the strengths and weaknesses of tests analyzing antithrombin and its molecular forms. Based on this information, a mass spectrometric test that molecularly characterizes antithrombin proteoforms was found to harbor the highest potential for improving the clinical pathway for ATD. The potential role of this test for improved patient management and outcome is discussed, and a future clinical pathway is envisioned in which molecular characterization of antithrombin by mass spectrometry offers a refined approach facilitating P5 healthcare for ATD.

Introduction

Even though pleads have been made for the introduction of precision medicine, the current healthcare system is still based on reactive medicine in which patients first have to become ill and are subsequently treated with therapies tailored towards the average white male [1]. This healthcare system is not durable nor optimal and instead P5 (preventive, personalized, predictive, participatory, psychocognitive) medicine has been proposed as an alternative to reduce the disease burden in the general population and hamper the surge in healthcare costs [2, 3]. Impact of P5 medicine is expected through prevention of disease, lowered time to accurate diagnosis, and optimized therapies in which response to therapy is increased [4].

Laboratory medicine plays an essential role in the identification of diseases and patient management. Recently, the definition of laboratory medicine was established as: "a clinical science and discipline, devoted to the quantitative measurement, or qualitative assessment [...] for either medical or research purposes. The results of these measurements are translated into actionable information for improving the care and/or maintaining the wellness of both a single individual and an entire population" [5]. The importance of laboratory medicine is clearly illustrated by a study finding that in a cardiology and oncology setting 66 % of clinical decisions are affected by in vitro diagnostic (IVD) tests, with application of IVD tests in 88 % of the initial diagnoses [6]. For medical tests to provide actionable information their test purpose should be clearly defined. This may entail setting a diagnosis, screening, risk stratification, and treatment selection and monitoring, highlighting the broad applicability of laboratory diagnostic tests [7]. To facilitate the transition to P5 medicine, laboratory testing must be advanced to generate information of sufficient depth thereby enabling risk stratification and accurate patient diagnoses.

Current laboratory tests in diagnostic pathways for thrombotic diseases lack analytical and clinical specificity to provide information of sufficient depth to enable P5 medicine. Specifically, this also holds true for antithrombin deficiency (ATD) testing, and thrombophilia screening in general, where the utility of diagnostic tests is being questioned [8, 9]. Several reasons have led to this skepticism: the relatively low prevalence of thrombophilia's, the lack of evidence that thrombophilia testing can prevent thrombosis in individuals lacking a history of thrombosis, and the limited effect of thrombophilia testing on treatment strategy [10-13]. For ATD, this is further complicated by the questionable clinical performance of the current first line screening test to identify ATD [14]. As a consequence, this test is more often applied as a liver function biomarker, as antithrombin (AT) is produced by the liver, than for thrombophilia screening [15, 16]. Because the first line AT test is not fit-for-purpose, personal and familial history and presence of factors provoking thrombosis play a large role in ATD diagnoses. It is known for ATD that thrombosis risk is heterogeneous and depends on the specific subtype [17], which cannot be provided through anamneses. A laboratory diagnostic test that can differentiate low- and high-risk patients would be required to optimize patient management by providing more accurate ATD diagnoses, guide (future) targeted therapies, and prevent recurring thrombotic events.

For a laboratory test to be successfully incorporated in a clinical care pathway, it must lead to proportionate clinical benefit. The European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) has proposed a test evaluation framework [18] in which five key components of in vitro tests are outlined. Ordered from development to clinical implications, the interrelated components are analytical performance, clinical performance, clinical effectiveness, cost effectiveness and broader impact. Newly developed tests should be evaluated for each of the five key components. Moreover, it is imperative to define the test role and test purpose of a newly developed test. To define the test role and test purpose as well as requirements for analytical and clinical performance of an AT test that enables P5 medicine it is imperative to understand the analytes molecular, biological, and clinical variation.

The aim of this manuscript is to compile the information required for the development of a

next-generation test for AT. It provides a narrative review of the aspects of AT and ATD relevant to understand and improve the clinical care pathway, current testing strategies for AT and ATD, and the application of mass spectrometry for molecular characterization of proteins. Lastly, we discuss the outlook of a novel mass spectrometry-based test for molecular ATD diagnostics in the 21st century.

Deficiency

Antithrombin plays a pivotal role in the regulation of the coagulation cascade. The deprecated name for AT, antithrombin III, stems from the misunderstanding that multiple forms of AT exist, each with its own role in the coagulation cascade [19, 20]. Although further research revealed a single true AT protein, the idea of having multiple (proteo)forms of AT is still actual to date, albeit not based on varying roles, but due to the presence of mutant or atypical AT proteoforms. A minimal amount of functional AT is required to sustain life, and a complete deficiency is therefor lethal *in utero* [21]. However, AT proteoforms may have varying functionality. For instance, in healthy individuals already two proteoforms of AT exist, namely α-AT and β-AT, of which the main α-proteoform accounts for ~ 90 % of the total AT [22]. However, mutations and post-translational modifications give rise to many more proteoforms, as will be discussed later.

Antithrombin deficiency (ATD) is a clotting disorder caused by a low concentration and/or dysfunctional AT. Although often believed to have a fairly low prevalence rate between 1:2000 and 1:5000, these rates were based on assumptions rather than actual studies [23, 24]. The observed prevalence is in fact much higher in studies conducted in apparently healthy donors (1:400 to 1:600 [25-28]), and is further increased in the venous thromboembolism (VTE) population (0.5 to 8:100 [29-32]). Of note, these prevalence rates were based on functional AT activity tests which are known to underdiagnose specific types of ATD and therefore true prevalence rates may exceed these numbers. ATD patients have the highest risks of thrombosis (odds ratio of 14 to 16) among inherited thrombophilia patients, highlighting the importance of correctly diagnosing and treating ATD [33, 34]. Patients are currently diagnosed for ATD by routine chromogenic AT activity tests, and even patients showing only a slight decrease of AT activity show an increased risk of thrombosis [35, 36]. Furthermore, specific mutations in AT are known to have a pathogenic phenotype but do not lead to lowered activity test results [14].

Molecular characteristics and structure of AT

The gene for AT, *SERPINC1*, encodes for 464 amino-acids. This includes a 32 amino-acid signal peptide which is essential for correct post-translational processing and is cleaved off prior to secretion, resulting in a mature protein of 432 amino-acids [37, 38]. Regarding post-translational modifications, AT contains four N-glycosylation sites (Asn-128, Asn-167, Asn-187 and Asn-224), of which one site (Asn-167) naturally shows varying occupancy. The type of glycan attached at these sites is highly conserved, with 95 to 99 % of the glycans being biantennary complex-type glycans [39]. The main (glyco)proteoform of AT, α-AT, is fully glycosylated, whereas the second form, β-AT, lacks the glycan at position Asn-167 [22]. The β-AT proportion of healthy donors was found to lie between 5.9 and 10.7 % [40]. Functionally, β-AT has an increased affinity for heparin leading to higher functionality [22]. This may be explained by the glycan interfering with the heparin-induced conformational change of α-AT from the inactive to the active form, or alternatively by steric hindrance of the heparin binding site by the large glycan moiety [22]. Levels of β-AT are elevated in young children, which is suggested to compensate for lower overall AT concentrations in infants [41, 42]. Furthermore, β-AT was found to play a key role in protection from vascular injury in a rabbit model [43, 44]. Recently, a study showed that β-AT may also play a role in ameliorating certain types of ATD as mutant β-proteoforms, in contrast to mutant α-proteoforms, retain their functionality despite the presence of HBS mutations [45]. Thus, β-AT appears to have high clinical relevance, even though this proteoform is currently not specifically evaluated by diagnostic tests.

A second naturally occurring proteoform has been suggested to be of clinical relevance: latent AT, a conformer of native AT. Latent AT is characterized by insertion of the reactive center loop into the A-β-sheet, leading to an irreversible inactive state which is unable to form a complex with a target protease, FIIa, and has low heparin affinity [46]. The latent proteoform is highly thermostable (with melting temperatures of up to 127 °C compared to 60 °C for native AT) and resistant to denaturation by high concentrations of urea [47, 48]. This proteoform was found to circulate in healthy donors at highly stable percentages of around 2.6 % ± 0.9 % of the total AT pool [48], likely owing to the metastable confirmation of native AT resulting in natural formation of the latent proteoform. Increased levels of the latent proteoform have been found during sepsis [48], and in patients with specific mutations in AT, such as p.Met283Val, p.His401Tyr, p.Pro439Thr, and p.Pro461Ser [49]. As slight increases in the temperature may facilitate the transition to the latent proteoform, the presence of increased levels of latent AT during fever may lead to thrombosis, both in ATD patients with mutations leading to high latent AT levels as well as in the general population [48, 50].

Serpins, the superfamily of proteins to which AT belongs, all have a similar structure containing a reactive site which determines their substrate specificity [37]. For AT, this site is positioned from Gly-411 to Asn-430 with the bond between amino acids Arg-425 and Ser-426 being the cleavage target of proteases [51]. Cleavage of this site is followed by a conformational change in AT leading to the inactivation of the protease [52]. Besides the reactive site, AT contains a heparin-binding site which involves positively charged residues, lysines and arginines, located across the AT molecule [53]. Interestingly, the length of the heparin polysaccharide influences the affinity of AT for specific proteases [54]. Furthermore, the mechanism by which the two main target proteases, Factor Xa and FIIa, interact with AT also differs. Whereas the affinity of FXa is highly dependent on the heparin dependent conformational change in AT, FIIa relies mostly on a so-called “template” effect in which heparin acts as a scaffold for the AT-FIIa-heparin complex to form [55, 56].

Subtypes of antithrombin deficiency

Hereditary ATD is subdivided into two main types, type I ATD, encompassing quantitative deficiencies, and type II ATD, encompassing qualitative (functional) deficiencies, both caused by mutations in the *SERPINC1* gene [57]. Currently, over 350 different mutations in AT are known, the majority being point-mutations [58, 59]. For patients with type I ATD, the mutation is always present in a heterozygous state and hinders the translation and/or secretion of the mutant proteoform leading to reduced concentrations of wildtype AT being present in circulation compared to healthy individuals. Type II ATD is caused by mutations that affect the functionality of the protein but do not (fully) omit translation and secretion, leading to a mixture of wildtype and variant proteoforms present in circulation for heterozygous mutations or exclusively variant proteoforms present for homozygous mutations. Similar to type I ATD, type II ATD mutations are mostly present in a heterozygous state, likely caused by the detrimental clinical effects of homozygosity [21]. However, a small number of type II mutations with mild phenotypes in the heterozygous state may be present in homozygous state (such as p.Leu131Phe also known as AT Budapest III, or p.Arg79Cys also known as AT Toyama) and often lead to severe clinical phenotypes.

Beyond mutations in the *SERPINC1* gene, ATD may also be caused by aberrant or missing glycosylation, for instance caused by congenital disorders of glycosylation (CDG) [60]. CDGs are a collection of genetic disorders affecting the overall glycosylation machinery and impacting many glycoproteins including AT [61]. Furthermore, specific mutations in AT have been associated with hypoglycosylation, namely p.Asn224His and p.Glu227Lys, which affect the N-glycosylation site at position Asn-224. Patients with these mutations presented with early and recurrent thrombosis, even though AT activity tests showed normal results [62]. With many variations possible in AT, it is not surprising that the clinical

phenotype of ATD is highly heterogeneous, and that identifying AT deficiency can be challenging for certain subtypes.

Current clinical pathway

Recommended testing strategy

Due to the complexity of ATD, there is no single test available that analyzes all clinically relevant aspects of AT, namely its activity, concentration, and molecular characteristics. Instead, various diagnostic tests are available, each analyzing an individual aspect of AT. A guideline on the diagnostic pathway was established by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (ISTH) which recommends a combination of activity and antigen tests in the diagnosis and subtyping of hereditary ATD [63]. The guideline, in agreement with general practice, recommends to first perform an activity test, as this test identifies (most) type I as well as type II ATDs. Activity tests may be influenced by interfering compounds (such as anticoagulants) and under certain conditions, thus repeat testing is required to exclude a transient state of ATD. To determine the subtype of ATD, a low activity result should be followed up by an antigen test. Low results on both tests indicate a type I ATD, while low activity and normal or slightly decreased antigen levels indicate a type II ATD. Of note, the ISTH guideline does not state when the testing strategy may be applied, which is a controversial topic and will be discussed later ("Variation in guidelines"). Taken together, the current strategy for identifying ATD is a rather blunt approach that oversimplifies the complexity of ATD, does not provide molecular insight, nor facilitates a basis for precision diagnostics and treatment.

Activity tests

Being the first-line test for diagnosing ATD, a wide range of commercial CE-IVD marked AT activity tests are available from different manufacturers. The tests assess the functionality of the total AT pool present in patient plasma. All tests have a similar design, with only minor changes since the development of AT activity tests in the 1970s [64]; citrate plasma is mixed with an excess of AT target protease, forming a complex with AT, after which the remaining target protease cleaves a chromogenic substrate leading to a color-reaction [65]. Depending on the substrate a specific optical density is measured, the height of which is inversely correlated with the amount of AT present in the sample. To interpret the OD, it is suggested to generate a calibrant based on a large pool of healthy donors (N ~ 100) and set the value thereof at 100 % activity. Although small fluctuations occur between laboratories, values between 80 and 120 % are generally considered healthy. The test is easy to use and can be applied on automated coagulation analyzers, enabling a rapid and inexpensive means of screening for ATD.

The various activity tests employ varying target proteases, either FIIa or FXa, of varying origin, either recombinant human or bovine. The use of either FIIa or FXa as a substrate may influence the sensitivity for specific types of ATD [66], as the mechanism by which AT inhibits these two target proteases also differs, as described previously. The target proteases added in the test can also be inhibited by other anticoagulant proteins to which end heparin is added to increase the functionality of AT and make the test more specific [67, 68]. As mentioned earlier, heparin acts as a co-factor for AT, greatly increasing its activity, and heparin chain length determines specificity of AT for the target protease. Heparin content in the various kits differs per activity test and the type of heparin is often not specified.

The variability between test kits leads to varying results depending on which kit a laboratory applies. Furthermore, the different subtypes that occur in ATD also behave differently within and between kits, depending on the specific mutation [14, 66]. It is therefore no surprise that the diagnostic sensitivity of the activity test differs between manufacturers and specific mutations. A study by Orlando et al. clearly demonstrated this by comparing the results of four different AT HBS mutations using four different activity tests **(Figure 1)** [14]. In this study only a single activity test (test B) could identify all four ATD HBS

mutations tested. Similar results have been found in other studies [66, 69-72], prompting the question whether the current testing strategy relying solely on activity tests as a means of diagnosing ATD is appropriate, or if alternative (molecular) options are required. Of note, good long-term performance of the activity tests was found in external quality assessment programs, with medians of 7.2 and 9.4 % for the within- and between-laboratory CV, respectively [73]. Such programs require large volumes of sample to supply all participating laboratories, entailing that the sample is often comprised of a large pool of donors and the level of the analyte of interest is artificially altered. Thus, these samples at best reflect the performance of activity tests for type I ATD, whilst the more problematic performance for molecularly heterogeneous type II ATD samples remains unexplored.

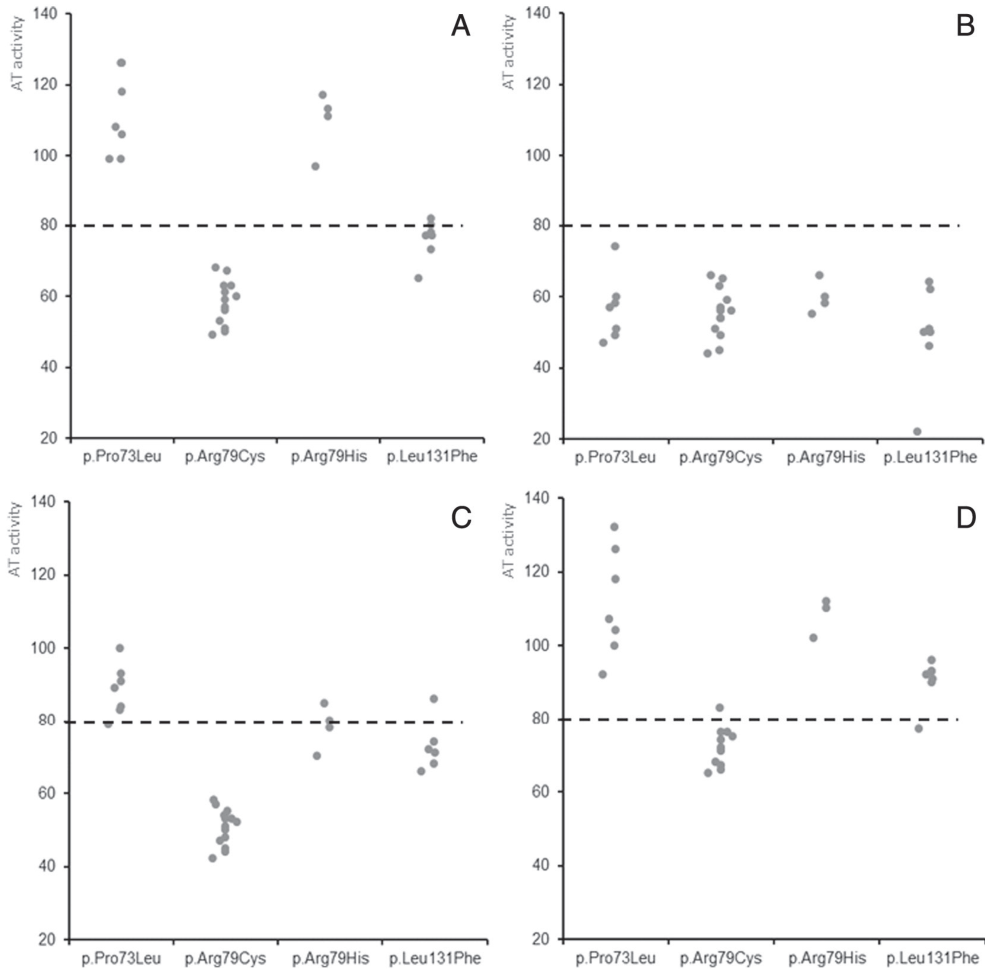

Figure 1. Antithrombin activity in 4 different heparin binding site mutations. A) HemosIL® Liquid AT, **B)** Innovance® AT, **C)** Coamatic® AT, and **D)** Biophen® anti-IIa. Dashed line represents lower limit of normal range. Reprint from Orlando et al., Thrombosis Research (2015) [14].

Antigen tests

Once ATD is diagnosed using the activity test, the antigen test may be employed as a follow-up test for low activity samples to classify the ATD subtype. Antigen tests provide a (relative) concentration thereby allowing for discrimination between quantitative type I and qualitative type II ATD deficiencies. There are various CE-IVD marked diagnostic tests to determine AT antigen, based on immunonephelometric

or (latex) immunoturbidimetric principles. This entails that antibody against AT is mixed with patient sample and the formed immune complexes containing AT are measured by light scattering or light intensity. Therefore, these tests rely "blindly" on the antibody employed, offer no molecular information, and consequently do not discriminate between AT proteoforms (or interferents). The readout of the antigen tests is often stated in units or % and are therefore by no means an absolute indication of the concentration of AT. To indicate the ATD subtype, one may divide the relative antigen result over the relative activity result and observe if an abnormal ratio is found compared to established reference intervals for both subtypes [67, 74]. Alternatively, one may interpret both values separately using their respective reference intervals thereby discriminating type I ATD, having below average values for both tests, from type II ATD, having normal values only for the antigen test. The first method is of course more sensitive in the correct identification of type II ATD mutations that may have both a qualitative and a (minor) quantitative defect. However, as AT subtyping does not necessarily influence patient management, antigen testing is only applied scarcely.

Sources and effects of diversity in antithrombin

Thus far, the diversity of the molecular features of AT has been discussed, whilst conversely the current diagnostic pathway for ATD is based on a simplified view that we may diagnose ATD in patients harboring varying molecular AT proteoforms by merely examining the overall activity of the total AT pool. To improve the diagnostics of ATD we must be aware of all aspects influencing AT levels. Therefore, the next section provides an overview of additional sources of AT diversity that can affect ATD tests and the performance of these test in the ATD clinical pathway.

Biological variation

Naturally, all components of the blood (or any biological fluid) have a fluctuating level, for instance due to changes in the production or consumption, cyclical influences, or because they are influenced by other biological processes [75]. For a clinical laboratory test to discriminate between a healthy individual and a patient harboring a disease, it is essential to know the variation of the analyte of interest in a healthy population, the so-called biological variation [76]. Specifically for the development of a diagnostic test, this information is necessary to establish the required analytical performance.

Many factors have been reported to affect the concentration and/or functionality of AT. Age is a known factor influencing the levels of AT [77-79]. During the first year of life, reduced activity levels of AT are found, with studies reporting values in neonates as low as 33 % [79]. Throughout childhood and adolescence, higher interindividual variation in AT activity values is found, although one study found the absolute values to be lower in children than in adults while two studies showed inverse results. Beyond activity levels, Ignjatovic et al. demonstrated that the molecular composition of AT is also different at a young age, with an increased contribution of the β-proteoform to the overall AT activity [41, 80].

Concerning gender, multiple studies have investigated the difference in AT activity between men and women with conflicting results. However, the most recent studies (published in the last 15 years) did observe significant differences [81-83]. These studies reported a lower level of AT activity in women of reproductive age compared to men, although the absolute difference was often not more than 2 to 3%. Interestingly, whereas the activity values of women tend to increase throughout life, the levels in men start to decrease around 40 years of age leading to men having lower values of AT activity than women at higher ages, with observed absolute differences of 4 to 9 %. This gender difference may be attributed to the influence of hormones, all the more as it is known for women that hormones influence AT activity. For example, oral contraceptive use is a known hormonal factor influencing AT activity [63]. Throughout life, women taking oral contraceptives have lower AT activity values of around 3 to 4% [81, 82]. Of note, this does not explain the lower AT activity values between men and women of

reproductive age, as a difference was also found when comparing men to women not on contraceptives [84]. After menopause, AT activity levels are around 2 to 3 % higher than in pre-menopause women. In pregnant women, it was found that AT activity levels lower approximately 10 to 20 % during the course of pregnancy [85, 86].

Taking these variations into account, it was suggested to establish specific reference intervals for age and gender groups [78, 82]. However, beyond the notion that AT is decreased in neonates and pregnant women, adjusted reference intervals are not common practice in clinical laboratories. Interestingly, a study by Goldman-Mazur et al. (2019) found a significant increase in the detection of ATD in men suspected of having a thrombophilia (due to clinical indication) aged > 50 years versus men < 50 years [87]. This could either indicate that the decreased levels of AT found in older men lead to an increase of clinical events, or that ATD is overdiagnosed in this specific group. Although the observed absolute differences in age and gender were not major in adults (the largest observed difference was 102.2 % activity for males versus 111 % activity for females aged 55-59 [84]), specifically for borderline AT activity values these differences could lead to over- or underdiagnosis.

Although this more concerns the prevalence of ATD, it is important to note that ATD prevalence may differ depending on ethnicity and nationality. For example, a study reported an increased prevalence of ATD in VTE patients among Thai, Chinese and Japanese ethnic groups compared to Caucasians [88]. Furthermore, it is known that certain mutations occur in high prevalence in specific regions, such as AT Basel in Finland and AT Budapest III in Hungary [89, 90].

Biological variation may lead to differences in the analytical performance required for a diagnostic test. According to the Milan consensus, diagnostic tests should comply with predefined analytical performance specifications which, ideally, should be based on outcome studies assessing the effect of analytical performance on patient outcome [91]. However, as these are not available for ATD, the alternative is to use biological variation data to establish these specifications. Fortunately, the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) working group on Biological Variation maintains a database containing information on the biological variation observed in laboratory measurands [92]. For both the AT antigen (**Table 1)** and AT activity test (**Table 2**), biological variation data is available, allowing calculation of the total allowable error of these the tests.

Table 1. Biological variation values of the AT antigen test as published by the EFLM Biological Variation Database.

Specification	CV_i	CV_g	imprecision (CV_a)	bias	MAu	Total Error
Minimum			5.4	3.3	10.8	12.2
Desirable	7.2	5.0	3.6	2.2	7.2	8.1
Optimal			1.8	1.1	3.6	4.1

Values were based on based on [93]. CV_i = within-subject variation, CV_g = between-subject variation, CV_a = analytical variation, Mau = maximum allowable measurement uncertainty. Accessed 28th of June 2024 [92].

Table 2. Biological variation values of the AT activity test as published by the EFLM Biological Variation Database.

Specification	CV_i	CV_g	imprecision (CV_a)	bias	MAu	Total Error
Minimum			2.5	3.2	5.1	7.4
Desirable	3.4	7.8	1.7	2.1	3.4	4.9
Optimal			0.8	1.1	1.7	2.5

Values were based on based on [85, 93-100]. CV_i = within-subject variation, CV_g = between-subject variation, CV_a = analytical variation, Mau = maximum allowable measurement uncertainty. Accessed 28th of June 2024 [92].

Pathologic variation

Beyond biological variation found in the normal population, several pathological states also bring along variation in AT. For instance, there is a clear link between low activity values and liver diseases such as liver cirrhosis, liver carcinoma, and hepatitis, which can be explained by the liver being the site of production for many coagulant factors including AT [101-105]. Furthermore, fever may lead to a reduction in AT activity as lowered levels were found during bacterial and viral infections [106-109] as well as during sepsis [110], potentially explained by an increase of the latent AT proteoform [111]. Furthermore, various pathologic aspects have shown contradicting results. Obesity has often been linked to lowered AT activity values and increased incidence of ATD [112-115], although contradicting studies showed inverse or insignificant results [116-118]. Similar conflicting results were found for both type I and type II diabetes mellitus [119-122]. However, this does indicate that caution should be taken when interpreting activity results from patients with metabolic disorders. Bowel disorders such as ulcerative colitis, irritable bowel disease and Crohn's disease have also been mentioned to alter AT activity values although once again there are conflicting reports stating increased, decreased, or similar levels in patient groups versus controls [123-126].

Although not directly caused by a disease state, various medications may also alter AT levels. Reports have mentioned an effect of heparin therapy, asparaginase and antipsychotics [127-129]. Furthermore, direct oral anticoagulants (DOACs) are known to influence results of AT activity tests, which, depending on the DOAC and the factor in the test, may lead to increased results [130]. It is therefore critical that in patients taking DOACs samples are taken after clearance of the medication, which may be challenging if patients are on anticoagulation due to a thrombotic incident, or that reversal agents are used prior to testing [131, 132].

Clinical symptoms: thrombosis

Variation in biological and pathological states leads to differences in AT activity values, but conversely the molecular diversity in AT also leads to variation in the clinical effects of ATD. Venous thromboembolism (VTE) is a well-established pathogenic consequence of low quantities and/or dysfunctional AT, with a 14 to 16 times increased risk compared to non-deficient individuals [33, 34]. In fact, the high occurrence of VTE in a single family gave the first indication that thrombosis risk could be caused by a genetic factor [20]. The co-occurrence of low AT activity in the same family linked ATD to thrombosis. However, although in general ATD leads to a high risk of VTE, this is heavily influenced by the ATD subtype. Type I ATD is known to lead to a high risk of VTE, with reports indicating that 60 to 86 % of patients suffer from VTE [17, 74, 133]. Type II ATD is generally considered to be more benign, although one study found type II RS as well as type II PE ATD to show risks similar to type I ATD [34]. A second study even found a higher risk of (spontaneous) VTE in type II RS ATD patients compared to type I ATD patients [133]. The overall lower risk of type II ATD is thus mainly attributable to type II HBS, although within this subtype high heterogeneity between specific causative mutations is observed. Many mutations are linked to type II HBS ATD and combined with the relatively low prevalence of ATD (and perhaps the difficulty to identify type II HBS using current diagnostics) this hampers the inclusion of ATD patients with a specific mutation in studies for in-depth characterization of the risks of individual HBS mutations. Consequently, only a few studies could recruit sufficient patients with a similar genetic background to analyze mutation specific risks of thrombosis. These studies found VTE occurrence in patients with type II HBS mutations to range between 0 and 50 % for heterozygous mutations, even rising to 88.8 % for the homozygous type II HBS Budapest III mutation [17, 89, 133, 134].

Clinical symptoms: alternative clinical phenotypes

Although there is an undisputable link between ATD and VTE, additional clinical implications of ATD have been reported but remain controversial due to lack of evidence. For instance, arterial

thromboembolism has been reported to occur in increased frequencies in patients with specific mutations, such as AT Basel (p.Pro73Leu)[134], AT Cambridge II (p.Ala384Ser)[135], and AT Padua (p.Arg79His)[14]. A meta-analysis investigating the risks of ATD in general reported an increased OR of 1.25 for arterial ischemic stroke [136], although this was not found to be significant likely caused by the lack of discrimination between subtypes and mutations. In contrast, a meta-analysis investigating the risk of arterial ischemic stroke in a pediatric population with thrombophilia found a significant OR of 3.29 for ATD [137, 138]. Thus, although controversial, arterial complications should not be disregarded in the context of ATD.

Recurrent pregnancy loss and obstetric complications are often mentioned to be related to ATD. Both type I and type II ATD were found to lead to high risks of obstetric complications [89, 139-142], with increased attention to specific mutations such as AT Basel (p.Pro73Leu) and AT Budapest III (p.Leu131Phe). As expected, as pregnancy is a provoking factor, studies describe a high incidence of thrombosis in pregnant women with ATD, with observed VTE occurrence ranging between 10 to 33 % depending on the mutation and whether the mother has a history of VTE. This is in line with a previous finding that in 12.0 % of the women experiencing VTE during pregnancy ATD was diagnosed [143], indicating that ATD plays a major role in pregnancy associated VTE. Moreover, an increased incidence of obstetric complications such as placental abruption or (late) pregnancy loss was also reported, with complication rates between 29.4 and 50 %, once again depending on the mutation and exacerbated in mothers who had previously experienced VTE. However, similar to arterial complications, the majority of evidence is limited to small studies. In an attempt to come to a strong conclusion, systematic reviews have tried to pool the relatively larger studies (often still comprising of less than 100 participants with various thrombophilia's). These reviews do indicate an effect of thrombophilia on pregnancy outcome, although they still state that evidence is too weak to provide strong recommendations [144, 145]. Therefore, current guidelines advise against thrombophilia screening in women experiencing recurrent pregnancy loss [146]. Similarly, treatment strategies for women with thrombophilia experiencing obstetric complications are controversial, with small case studies reporting favorable outcomes while larger studies, lacking patient stratification, advise against the use of prophylaxis to improve pregnancy outcomes [147, 148].

Taken together, the assumption that ATD is merely involved in the development of thrombosis is unlikely and if there is one thing that all studies agree on, it is that more research (and better research methods) into ATD and thrombophilia's in general is crucial.

Variation in guidelines

Clinical pathways for hereditary thrombophilia's, as is the case for ATD, suffer from a low amount of evidence to guide clinical decision making. For ATD specifically, this is largely attributed to the fact that larger studies on (hereditary) thrombophilia's do not include sufficient patients with ATD to generate strong evidence concerning ATD. Furthermore, these studies identify patients based on general screening tests for thrombophilia's such as the AT activity test, which is known to miss certain subtypes of ATD and does not give molecular insight. This leads to an underrepresentation of ATD in general as well as a low variety of ATD subtypes in studies on which guidelines are built. Furthermore, as described previously, "the general ATD patient" does not exist when reflecting on the large variation found between ATD individuals. Thus, generalized approaches for the screening of all persons suspected of having ATD or a general treatment for ATD patients is likely not appropriate.

However, in our current healthcare system, even if evidence is lacking, guidelines are still being established. The most recent American Society for Hematology (ASH) guideline on thrombophilia testing is comprised of 23 recommendations, of which only 1 was a strong recommendation and all others are stated as suggestions due to low amount of available evidence [149]. The British Society for

Hematology also provided a specific guideline on recommendations for when testing a deficiency of natural anticoagulants, such as AT, is warranted. This guideline stated to only test if clinical management would be impacted although, similar to the ASH guidelines, the level of certainty in the evidence is low [150]. Similar issues are found in the treatment of VTE, with major guidelines providing, sometimes conflicting, recommendations on the (prevention and) treatment of VTE in the context of surgery or pregnancy based on weak evidence for the majority of recommendations [151, 152].

Guidelines provide recommendations based on the general patient and cost effectiveness of testing and treatment. In contrast, individual clinicians are inclined to test patients or family members in situations not recommended by these guidelines or treat them based on their own clinical experience on ATD patients [153-155]. Varying views on the value of a thrombophilia diagnosis has led to varying clinical pathways for screening in institutions diverging from major guidelines such as the ASH guideline [153, 156, 157]. Beyond the views of individual clinicians, the concept of P5 healthcare entails empowerment of our patients and giving them the right to know about their health status, thereby transforming them from bystanders to active decision-makers [158]. Thus, if we want to transform the ATD clinical pathway to a P5 healthcare approach, we must generate more information on the individual patient-level, instead of focusing on generalized concepts of ATD or thrombophilia.

Influence of diversity on the current clinical care pathway

If one thing is clear from the currently observed and oftentimes unexplained diversity in AT and ATD, it is that fifty years of AT research employing mostly the functional AT activity test has not provided us the evidence to sensitively and specifically diagnose ATD patients, prevent VTE (or other disease) occurrence nor treat patients optimally. The scraps of evidence on which current guidelines and treatments are based on has led to an unrefined clinical care pathway (**Figure 2**). To improve this pathway, we must better understand AT and ATD, and find approaches to implement this new knowledge in clinical care.

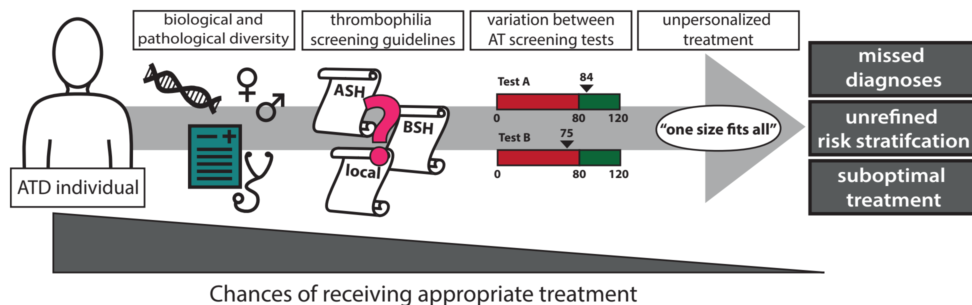

Figure 2. Overview of the influence of components of diversity on the clinical pathway for ATD patients. ATD patients are inherently diverse due to genetic, biologic, and pathological factors. Furthermore, the decision on when to screen for thrombophilia's differs depending on the guideline a healthcare setting adheres to, or the clinicians own interpretation. If screening is employed, diversity of screening tests may lead to missing diagnoses, depending on which test is employed. Lastly, even if patients are correctly diagnosed, the current treatment strategy is still a generalized anticoagulant treatment without considering the molecular basis of the ATD. Together, this diminishes the chances that ATD patients are treated appropriately, and VTE recurrence risk or overtreatment of the ATD individual may persist.

Alternative testing options

Most studies into ATD have employed AT activity tests, as these are commercially available and easily applied in large cohorts. However, the low level of information gained from activity tests hampers stratification of patients in these cohorts, nor do they identify all patients with ATD. To this end, specialized research into AT has often resorted to alternative techniques that are more labor intensive but provide additional information on for instance the heparin affinity or glycosylation status of

certain proteoforms (**Figure 3**). Although these techniques offer valuable information in the research setting, their translation to patient diagnostics is often not possible, limiting their usefulness. Only two techniques have succeeded in progressing from research use only, to their applicability in patient diagnostics, namely genetic screening, and LC-MRM-MS analysis. An overview on all techniques, alternative to the standard activity and antigen test, currently applied in AT research and (if possible) diagnostics will be provided in this section to provide an overview of the strengths and weaknesses of each test and generate insight into which (molecular) aspects of AT can and should be investigated.

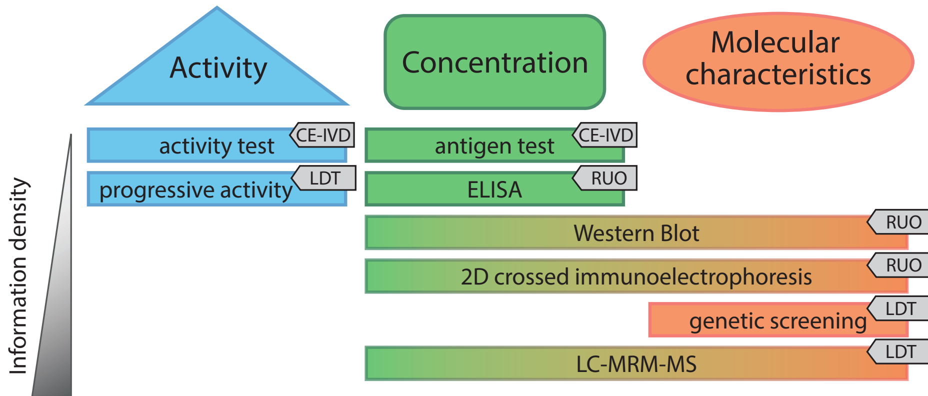

Figure 3. Test options for antithrombin. Overview of available options to investigate antithrombin, categorized by which aspect of antithrombin the test focuses on. CE-IVD = conformitee européenne in vitro diagnostics, LDT = laboratory-developed test, RUO = research use only. Figure is adapted from Kruijt et al. (2024) [159].

Research-only methods

In the research setting, multiple techniques are available to investigate AT, as there are often no stringent constraints on analytical performance or sample throughput. Historically, rocket immunoelectrophoresis was often applied to investigate the AT concentration of patient samples, enabling relative quantitation of AT [160]. The addition of heparin in the second dimension of the gel enabled exploration of the heparin affinity of the AT proteoforms in a sample and increased the information density of the test. Although labor intensive, the relatively simple execution and ability to gain functional information entails that the test is still being used to date in research settings [106]. However, it is not feasibly to process large numbers of samples or (routinely) incorporate this technique in a diagnostic pathway. Similarly, western blot is being applied in research settings to investigate the presence of proteoforms in clinical samples as well as in recombinant study samples [62]. Similar to immunoelectrophoresis it is a labor-intensive technique but does provide insight on the number of proteoforms present in a sample, provided that they can be separated on gel (which may be troublesome for point mutations leading to very small mass differences). Western blot does enable information on different proteoforms due to altered mass or glycosylation, although it does not reveal which exact proteoform is present. For instance, western blot results showing altered glycosylation, based on mass shifts corresponding to the mass of a glycan or by applying de-glycosylating enzymes such as PNGase F as a control, indicate altered overall glycosylation but not the exact sites at which glycans may be absent [60]. However, studies investigating glycoproteoforms did enable valuable insight into the clinical relevance of AT glycosylation, albeit translation of this information into the diagnostic and clinical pathway has remained troublesome [60, 62]. Lastly, ELISA may also be applied to investigate the concentration of AT in samples, although this is the least informative research technique providing merely a concentration without information on the proteoforms. Furthermore, this technique is dependent on antibodies, which, combined with the indirect read-out of ELISAs, may lead to errors [161].

Progressive antithrombin activity test

The standard AT activity test lacks sensitivity but has the benefit of being easily applied in large cohorts or for diagnostic purposes. Thus, in an attempt to increase its sensitivity and make the activity test more valuable, Kovács et al. established an alternative chromogenic activity test, termed the progressive AT test. The test makes use of AT's dependence on heparin and the existing infrastructure and protocols for the standard AT activity test. In short, polybrene, a heparin neutralizer, is added to the sample dilution buffer and the incubation time of the reaction mixture is extended, allowing examination of AT activity without heparin acting as a cofactor [162]. The ratio of the progressive test versus a heparin-cofactor dependent test provides a ratio that discriminates between type I and type II ATD and could even distinguishing between homozygous and heterozygous p.Leu131Phe patients (a type II HBS deficiency). As the test requires a relatively simple adjustment of the standard activity test it may be executed in any laboratory that already executes AT activity tests to provide clinically relevant information in the absence of genetic screening [63, 67]. However, the test does not provide detailed molecular information on the mutation underlying the ATD, nor the glycosylation status, and certain HBS mutations were found to still be missed with this test [163]. Therefore, although currently a viable option to improve the ATD clinical care pathway while using the existing infrastructure, the progressive activity test is by no means a suitable candidate test to facilitate P5 healthcare.

Genetic testing

Genetic testing is the technique most commonly mentioned when precision medicine is discussed, largely owing to the success of genomic profiling in precision oncology [164]. However, in the field of thrombosis and haemostasis, the introduction of genetic testing has been limited almost exclusively to research and has only been sparsely applied in clinical practice [165]. It has been suggested to selectively genetically screen patients suspected of ATD [8, 63], specifically for mutations that are known to present with normal activity and antigen values, such as transient hereditary AT deficiencies caused by AT Dublin or AT Cambridge II mutations [166]. Furthermore, genetic testing can determine homozygosity of a mutation. Zeng and coworkers even recommended the inclusion of genetic screening as a first-line test upon suspicion of hereditary AT deficiency, due to the risk of missing diagnoses [167]. However, as the exact mutation often does not lead to a change in treatment strategy, genetic testing is only sparsely applied and not always available. More importantly, genetic information cannot predict the expression and secretion of AT proteoforms into the circulations, nor can it identify alterations in post-translational modifications, such as the glycosylation of AT, even though the latter was found to be clinically relevant [60]. Thus, it appears that the hype surrounding genetic screening in oncology is not translated to the field of thrombosis and haemostasis and instead alternative molecular techniques should be evaluated.

Multiple-reaction-monitoring mass spectrometry

Precision medicine is based on the concept that clinical care should be tailored to the individual patient, thereby optimizing patient management. To accommodate a precision medicine approach, focusing merely on genetics does not suffice, as post-translational modifications, physiology, and environmental influences introduce additional variation at the patient-level [168]. Proteins, in contrast to genes, are modified by these additional factors and actively play a role in health and disease. Therefore, proteomics and specifically the measurements of proteoforms has been put forth as a more refined technique to understand human biology and improve disease diagnostics [168, 169]. Currently, the measurement of proteins in clinical chemistry relies heavily on immunoassays, despite potential flaws such as discordance between tests, interference by auto-antibodies, and hook effects [170]. Furthermore, immunoassays offer limited insight into proteins, as they often only allow quantitation but no molecular information. Instead, mass spectrometry (MS) is the most omnipotent technique as it enables quantitation, identifies mutations, and monitors post-translational modifications. To

this end, MS has been proposed as a potentially revolutionizing technique in the clinical laboratory and for the application of precision diagnostics [170, 171]. Specifically, multiple-reaction-monitoring (MRM-)MS currently holds the greatest potential due to its high sensitivity and specificity, two critical qualities for the development and validation of clinical diagnostic tests [172].

Multiple-reaction-monitoring MS is based on the measurement of surrogate peptides of the protein of interest using so-called "transitions", which is the combination of precursor peptides and related fragment ions [173]. To form peptides, proteins are first digested by a sequence specific enzyme, most often trypsin, to form smaller and analytically simpler peptides unique to the protein of interest [174]. To measure these peptides, triple-quadrupole MS instruments are used, which filter for the precursor peptide mass-to-charge (*m/z*) value in the first quadrupole, fragment this peptide in the second quadrupole, and filter for the fragment ion *m/z* value in the third quadrupole. If carefully validated, this peptide/fragment pair is essentially a fingerprint of the protein of interest and provides high selectivity, which may be further increased by measuring multiple (e.g. three) transitions per peptide of interest and combining this MRM-MS with liquid chromatography to monitor the transition of interest at a specific retention time [175]. As the triple-quadrupole instrument filters for the transitions of interest and only these are passed to the MS detector, high sensitivity is achieved [172]. Mass spectrometry is not inherently quantitative, as performance of the instrument may fluctuate between measurements, but the addition of internal (peptide) standards allows for the correction of these fluctuations, thereby enabling accurate and robust quantitation [176, 177]. Beyond quantitation, transitions may be developed that target mutations instead of wildtype peptides or include post-translational modifications such as glycosylation [172, 178-180]. This entails that MS-based tests can generate both quantitative and qualitative information, which is ideal to accommodate precision diagnostics.

However, the application of MS for the monitoring of mutations or post-translational modifications has been mostly applied in discovery proteomics and only sparsely in clinical proteomics. Even the translation of protein biomarkers to quantitative clinical chemistry proteomics faces many challenges, such as medical utility, clinical feasibility and ensuring robustness [181]. Therefore, extensive validation is required, which can already be a hurdle for MS tests that only provide quantitation of a protein of interest [182, 183]. Ideally, a new test should be developed according to the test evaluation framework proposed by the EFLM to ensure that the test provides added benefits to the patient, either in the form of improved health outcomes or other aspects such as reduced time to diagnosis or lowered costs [18]. By adhering to these guidelines, it has been shown that when a test has been thoughtfully developed, validated according to predefined analytical performance specifications and the performance is continuously monitored, long-term robustness of quantitative MS-based tests is possible [184]. Thus, for proteins existing in diverse proteoforms with clinical relevance we should pioneer into the new clinical chemistry field of quantitatively and qualitatively measuring proteoforms to enable precision diagnostics. Antithrombin is a prime example of a protein existing in clinically relevant proteoforms, for which the current clinical tests are underperforming, and to which end an MS-based diagnostic proteoform test may be the key to unlocking a precision medicine approach.

Outline of this thesis

Antithrombin is *in nature* a highly diverse protein, existing in many proteoforms and displaying highly variable functionality leading to molecular, biological, and clinical variation. Current first-line diagnostic tests for ATD focus on measuring the activity of the overall pool of AT proteoforms in a patient instead of differentiating between and characterizing the highly diverse AT proteoforms present. By reporting the average activity of the total AT proteoform pool, diagnostic uncertainty may arise and ATD subtypes may be missed. This "*imprecision diagnostics*" approach entails that we are harming our patients as we are overlooking the pathological diversity of ATD. Note that Precision Diagnostics and

personalized healthcare instead embrace the complexity and enable better patient stratification and tailored treatment to improve the quality of healthcare, as is already applied in Precision Oncology [170]. However, thrombosis research is still largely based on crude, non-harmonized functional tests with synthetic substrates, which work well ***on average***. Therefore, with this thesis I aim to investigate the potential of next-generation protein diagnostics by mass spectrometry in the context of ATD. The development of the next-generation test for AT was in accordance with the European Federation of Clinical Chemistry and Laboratory Medicine's test evaluation framework [18]. Specific objectives of this thesis include the development and analytical validation of a mass spectrometry-based test that measures AT proteoforms in a clinical laboratory setting, as well as investigating the clinical validity and test role of this test.

In **this chapter**, the current knowledge on the molecular characteristics of AT and available tests for diagnosing and studying AT and ATD were described. Furthermore, this chapter highlighted the ignored and neglected but clinically relevant interindividual variability of AT and reveals the potential for improved patient management and patient outcome due to evolutions in science, technology and metrology. As a response to this, a liquid-chromatography coupled to multiple-reaction- monitoring mass spectrometry (LC-MRM-MS) test was developed and analytically validated, as described in **chapter 2**. The LC-MRM-MS test aims to investigate in as much detail as possible the molecular status of AT proteoforms in clinical samples. With the aim of applying such a test in a clinical setting, the analytical performance of the test was thoroughly investigated, encompassing precision, carryover, linearity, stability, and analytical specificity. Furthermore, a first glimpse into clinical results is given by the establishment of reference intervals based on healthy donors and a method comparison in transient ATD patients. **Chapter 3** describes the additional possibility of applying the test in other sample matrices than the analytically validated citrate plasma matrix, likely owing to the elimination of matrix effects by the immunocapture step present in the test.

The LC-MRM-MS test generates a large volume of data points per analyzed sample. Thus, for successful application of the LC-MRM-MS test results in the current clinical care pathway for patient management the data must be processed, and key elements extracted in a streamlined workflow. This workflow is therefore described in **chapter 4** to provide guidance to clinical chemists aspiring to apply similar approaches. **Chapter 5** then describes the molecular insight generated by applying the LC-MRM-MS test in a hereditary ATD patient cohort. Furthermore, it provides evidence on the scientific validity of applying the LC-MRM-MS test in diagnostic setting. **Chapter 6** explores a specific clinical case of a patient presenting with ambiguous AT activity results. This case describes, at patient-level, the current shortcomings of the diagnostic test/pathway and proposes an alternative pathway to minimize the underdiagnosis of patients.

Neglecting the molecular characteristics of AT and the distinct features of individual ATD mutations is hampering the establishment of better guidelines and treatment strategies. **Chapter 7** therefore illustrates how the LC-MRM-MS test for AT can molecularly characterize patient groups with a specific mutation, in this case the highly prevalent AT Budapest III mutation. To bring the thesis to a close, **chapter 8** describes an outlook on how this test may facilitate a precision diagnostics approach and provide a basis for bringing the diagnostics and clinical care for ATD into the 21st century.