

State-of-the-art testing for alpha-1 antitrypsin deficiency

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ABSTRACT

Background: Alpha-1 antitrypsin deficiency (AATD) is a genetic condition characterized by low serum levels of the protein alpha-1 antitrypsin. Because there are no unique clinical symptoms that point to a definitive diagnosis of AATD, laboratory testing is crucial to differentiate this disease from others.

Objective: To summarize advances in laboratory techniques used to test for AATD.

Methods: Data were sourced from a nonsystematic literature review of MEDLINE and the author's personal literature collection, and by checking reference lists of sourced articles.

Results: Since the original description of AATD by Laurell and Eriksson in 1963, testing methods have undergone major changes. Currently, alpha-1 antitrypsin protein is quantified by immunologic measurement in serum, and the phenotype is characterized by isoelectric focusing and/or targeted genotyping of predefined mutations. In addition, whole-gene sequencing of the gene SERPINA1 can be undertaken. However, this is costly and generally used only if targeted genotyping cannot conclusively identify the variant. The introduction of next-generation sequencing (NGS), which enables rapid and accurate sequencing of large quantities of DNA fragments in a single reaction, may help reduce costs. With its increasing availability, NGS may begin to appear in testing protocols. Clinical guidelines recommend that patients are tested for AATD if they have chronic irreversible airflow obstruction, especially those with early onset disease or a positive family history of AATD. Despite this, AATD is still underrecognized, and significant delays exist between symptom onset and diagnosis.

Conclusion: Traditional testing practices have limitations. Screening programs that incorporate NGS are the most comprehensive methods available for accurate diagnosis of AATD.

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Alpha-1 Antitrypsin deficiency (AATD) is a genetic condition characterized by low serum levels of the protein α -1 antitrypsin (AAT) (also known as α_1 -proteinase inhibitor). An important function of AAT is to inhibit neutrophil elastase in the lung. Neutrophil elastase is a proteolytic enzyme released by activated neutrophils during inflammation. The absence or deficiency of AAT can lead to an imbalance between elastase and antielastase activity. This accelerates degradation of lung tissue by neutrophil elastase, which may lead to development of chronic obstructive pulmonary disorder (COPD) and early onset emphysema during the third to fourth decade of life.¹ Tobacco smoking is a major additional risk factor. COPD is a group of progressive and debilitating respiratory conditions that result in a significant burden of disease, both medically and financially.^{2,3} Although

symptomatic treatment of COPD and/or emphysema is available, the only treatment that addresses the deficiency in patients with AATD, and has recently been shown to slow the course of the disease, is intravenous therapy with AAT.^{4,5}

In addition to lung complications, patients may also develop liver disease due to the aggregation of misfolded AAT protein within hepatocytes (where it is synthesized).⁶ Clinical presentation varies but can be seen as early as childhood. For severe liver disease, there is no known treatment beyond transplantation.⁶ Less common disorders associated with AATD include panniculitis⁷ and vasculitis.⁸ Early introduction of lifestyle changes, such as smoking cessation and the institution of appropriate therapy (*i.e.*, AAT therapy), may lessen and/or delay these deleterious health consequences. However, there are no unique clinical symptoms that point to a definitive clinical diagnosis of AATD. Typical respiratory symptoms, including wheezing, dyspnea, and shortness of breath, are common to other conditions, such as smoking-related COPD and asthma.⁹ Furthermore, it is now recognized that COPD and asthma can occur together, a condition known as COPD-asthma overlap syndrome.^{3,10} Therefore, accurate laboratory testing is of key importance in diagnosing AATD.

This narrative review aimed to summarize advances in techniques used to test for AATD since the condition was first described ~50 years ago. The review continues through to the present day and state-of-the-art

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genetic detection of numerous AAT variants. Data were sourced from a nonsystematic literature review conducted using MEDLINE with the following broad search terms with no date or time restrictions: “alpha-1 antitrypsin deficiency”[all fields] and sequencing[all fields]; and “alpha-1 antitrypsin deficiency”[title/abstract] and testing[title/abstract]. The author’s personal literature collection also was used, in addition to checking the reference lists of sourced articles.

GENETICS AND PREVALENCE OF AATD

AATD is caused by mutations in the *SERPINA1* gene that encodes the AAT protein. Mutations in this gene can cause either production of a misfolded protein, which may lead to its retention in the endoplasmic reticulum and reduced serum levels, or a complete absence of protein product (null phenotype). The traditional proteinase inhibitor (PI) nomenclature was first introduced by Fagerhol and Laurell¹¹ in 1967 and is still widely used today. The nomenclature identifies not only the known genetic variants of the AAT protein but also the continuously increasing number of new variants. Alphabetical abbreviations assigned to the different allelic variants denote their position in a pH gradient by isoelectric focusing (IEF). The wild-type functional allele is known as PI*M, and the two most common deficiency alleles are PI*Z and PI*S, which together account for >95% of all cases of AATD.^{12,13} Inheritance of AATD is codominant. Therefore, the functional level of the AAT protein is determined by both alleles. Individuals with two different alleles, *i.e.*, heterozygotes, will have AAT levels between the levels typically found for each allele individually. The PIZZ phenotype leads to large deficiencies in AAT, which result in serum levels between 10 and 15% of the normal phenotype (PIMM). PISZ, PISS, and PIMZ phenotypes cause serum levels between 35 and 70% of normal, depending on the combination of alleles.¹²

The PI*Z and PI*S deficiency alleles first arose in Europe.¹⁴ Today, AATD is most commonly found in individuals of white European descent. However, the Z and S alleles have also been identified in other racial groups, *e.g.*, black Africans and Arabic individuals.¹⁵ The prevalence of AATD varies widely according to geographic location. Northern and central Europe and North America account for 41 and 24% of individuals with the PIZZ phenotype worldwide, respectively.¹² In 2010, the number of Americans who carried either the S and/or Z allele was estimated to be 1 in every 17 individuals (5.8%), which equates to a total prevalence for the homozygous PI*ZZ genotype of 1 in 6211 individuals.¹⁶ Epidemiologic studies have concluded that AATD is one of the most prevalent single-locus genetic diseases worldwide. However, of the estimated 100,000 people currently living with AATD in the

United States, <10% are thought to have been diagnosed.¹⁷ This is partly because AATD lacks any identifying symptoms that are unique to this disease, and many patients with AATD are incorrectly diagnosed with COPD of other etiologies or other respiratory disorders.^{9,18} Furthermore, some individuals may be asymptomatic.

HISTORY OF AATD TESTING

The original description of AATD was based on the recognition of low levels of the AAT protein on conventional paper electrophoresis.¹⁹ This observation was further substantiated by immunoelectrophoresis and a functional assay that measures trypsin inhibiting activity.^{19,20} Since this time, AATD testing has undergone major innovations. With the application of immunologic methods to quantitate levels of AAT, including radial immunodiffusion, nephelometry, and enzyme-linked immunosorbent assay, it was possible to determine the serum level of AAT with a high degree of accuracy. However, these methods, which are based on the measurement of an AAT-antibody complex, were unable to distinguish a structural change in the deficient protein. Thus, they could not determine the underlying genetic variations and corresponding phenotype. This was resolved with the identification of a mutation that produced a major structural change in the protein and deficiency in AAT levels.²¹ At this time, acid starch gel electrophoresis was introduced into the analysis of AAT. When used in combination with antigen-antibody crossed electrophoresis, it showed, convincingly, that the deficient protein migrated electrophoretically differently from the normal (wild-type) protein and was present in a lower concentration.²²

CURRENT ROUTINE LABORATORY METHODS

Currently, almost all forms of severe deficiency can be identified by using quantitative analysis of AAT serum levels.²³ Severe deficiency is defined as an AAT level of $\leq 11 \mu\text{M}$ or 52 mg/dL.¹³ Individuals with AAT levels below this protective threshold (*e.g.*, PI*ZZ) are at an increased risk for early onset emphysema, particularly in those who smoke. However, it is important to note that there is wide variability in AAT serum levels for each genotype (Table 1). Therefore, quantitative testing alone is not sufficient to establish whether underlying genetic variations exist that could contribute to AATD and impact an accurate diagnosis.

Furthermore, AAT levels may overlap with the normal (PI*MM) genotype. AAT is an acute-phase protein, and serum levels may fluctuate in response to inflammatory disease, pregnancy, use of oral contraceptives, and vaccination.^{24–27} Ottaviani *et al.*²⁸ analyzed C-reactive protein (CRP) levels in a cohort of patients

Table 1 Serum levels of α -1 antitrypsin deficiency genotypes*

Genotype	Serum Level, mg/dL#
MM	105–164
MS	83–137
MZ	66–100
SS	73–106
SZ	49–66
ZZ	20–45
Null/null	0

*From Refs. 17, 54–56.

#Measured by using a typical commercial standard (mg/dL); a level of $\leq 11 \mu\text{M}$ (52 mg/dL) is associated with an increased risk for emphysema.

with AATD. Systemic inflammatory status was found to parallel an increase in levels of AAT. Overall, 80% of subjects with intermediate deficiency of AAT and a CRP level of $>8 \text{ mg/L}$ had an AAT level above the cutoff of 113 mg/dL .²⁸ Therefore, an acute-phase reaction may elevate AAT levels, which masks the underlying deficiency. Simultaneous determination of CRP and AAT levels may be useful in the correct diagnosis of heterozygotes that carry intermediate AATD genotypes. To this end, some AATD testing programs routinely measure CRP levels to determine if an individual is experiencing an acute-phase reaction at the time of testing.

When serum levels are shown to be low, phenotyping by IEF, the method that superseded acid starch gel electrophoresis, is commonly used.²³ IEF is a form of qualitative analysis that identifies protein variants based on their isoelectric position in a pH gradient gel (Fig. 1). IEF is suitable for routine diagnostic use and is usually performed in a reference laboratory. However, it requires skilled personnel to undertake the analysis and interpret the results. IEF is able to detect the most common variants of AAT, and although able to detect some rare variants, it does not on its own detect single null alleles, rare variants not routinely encountered, or M-like alleles.²³ A total lack of protein banding on IEF gels, in conjunction with a demonstrated lack of AAT in serum, confirms an individual with a null/null phenotype. Interpreting the banding patterns of heterozygotes that carry one null allele is difficult and may lead to an incorrect conclusion that the patient only has the normal (PIM) phenotype. Variants with overlapping banding patterns, which result from similar protein migrations, can also be difficult to distinguish.

In addition to IEF, qualitative testing includes targeted genotyping of predefined mutations by using polymerase chain reaction (PCR) based methods (e.g., Z [Glu342Lys], S [Glu264Val], and F [Arg247Cys]). In recent years, there has been an increase in the avail-

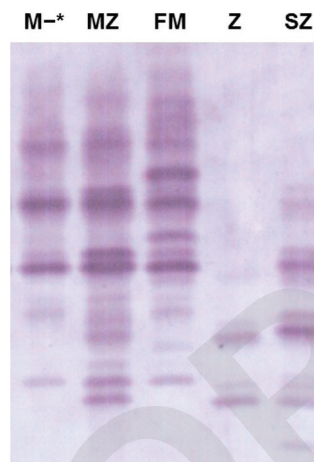


Figure 1. Isoelectric focusing of α -1 antitrypsin deficiency phenotypes (from Ref. 57). *Interpreting the banding patterns of heterozygotes carrying one null allele (M-) is difficult and could lead to an incorrect conclusion that the patient only has the normal (PIM) phenotype.

ability of commercial off-the-shelf DNA oligonucleotide probes that enable the identification of specific gene variants using real-time PCR. A large number of new mutations of the *SERPINA1* gene have been identified, often discovered while screening large population samples or when testing patients who have lung and/or liver disorders. Approximately 500 variants of the *SERPINA1* gene are known to date.²⁹ The use of real-time PCR to screen for novel or rare variants is costly because a separate set of primers is required for each allele. In practice, laboratories only screen for a small number of common mutations once the AAT serum level has been shown to be below normal.²³

The combination of serum levels with IEF and/or PCR as summarized above is able to diagnose $\sim 95\%$ of all individuals tested.³⁰ However, these methods are restricted in their ability to accurately diagnose individuals with rare and/or novel variants, and to differentiate between a homozygous and a heterozygous state with a single null allele (e.g., with IEF). Various testing algorithms have been proposed, all of which begin with serum AAT level testing, followed by either phenotyping and/or genotyping.³¹ The criterion standard method for detecting rare and/or novel variants is whole-gene sequencing in conjunction with serum AAT testing.²⁹ Gene sequencing has been used to accurately diagnose cases of AATD that had been misclassified when using a conventional testing algorithm.³²

NEW TESTING TECHNIQUES: NEXT-GENERATION SEQUENCING

In general terms, gene sequencing is an expensive testing method³³ and is used for the diagnosis of AATD only when targeted genotyping by PCR is unable to conclusively identify the variant.²³ Next-gener-

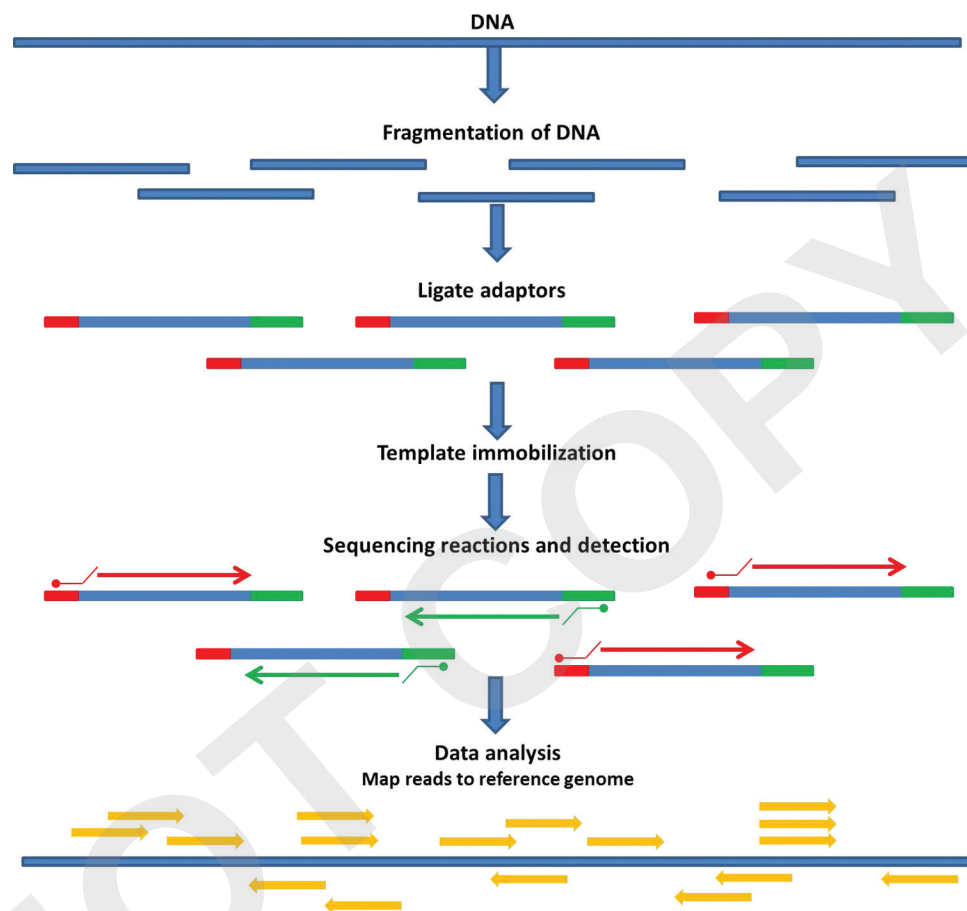


Figure 2. Next-generation sequencing schematic. Next-generation sequencing consists of template generation, sequencing reaction and detection, and data analysis, which thus allows the identification of rare or unknown variants, including single nucleotide polymorphisms, insertions and/or deletions, and copy number variants.

ation sequencing (NGS) enables rapid and accurate sequencing of large quantities of DNA fragments in a single reaction.³⁴ Irrespective of the specific methodology and equipment used, or the DNA region to be sequenced, the basic process of NGS is the same (Fig. 2). The source DNA is isolated, amplified, and sequenced, and then mapped against a reference sequence to identify genomic variations, including single nucleotide polymorphisms, insertions and/or deletions, and copy number variants. Benefits include the identification of mutations within noncoding regions, regulatory elements, and the promoter, which can lead to downstream variability in protein expression and the requirement for as little as 10 ng of DNA.

With its increasing availability, NGS has begun to appear in AATD testing protocols.³⁵ In recent years, NGS has been used to identify new variants and disease-causing mutations in a wide variety of diseases, including cardiomyopathy,³⁶ familial hypercholesterolemia,³⁷ Alport syndrome,³⁸ and myofibrillar myopathies.³⁹ Furthermore, as whole-genome or whole-exome sequencing becomes more widespread and is used as part of clinical care pathways in patients undiagnosed with a specific condition, there is a chance to detect so-called genomic incidental findings. Some of these incidental findings, in fact, may be well charac-

terized and linked to morbidities.⁴⁰ Reporting of incidental findings for AATD would aid accurate diagnosis in patients who might otherwise be misdiagnosed or missed altogether.⁴⁰ This would allow earlier disease management, including preventive measures such as smoking cessation or prevention, and avoidance of exposure to environmental pollutants. In addition, it could identify patients who may be suitable candidates for AAT therapy.⁴¹

TESTING RECOMMENDATIONS AND SCREENING

More than a decade ago, a bulletin from the World Health Organization⁴² and a joint statement from the American Thoracic Society and European Respiratory Society task force,¹⁷ recommended that the following at-risk groups undergo genetic testing for AATD: adults with symptoms of AATD and emphysema, COPD, or asthma with airflow obstruction not completely reversible with the use of bronchodilators; all individuals with unexplained liver disease; subjects without symptoms of AATD and with persistent obstruction found on pulmonary function tests, and identifiable risk factors; and adults with necrotizing panniculitis. Recently published recommendations are

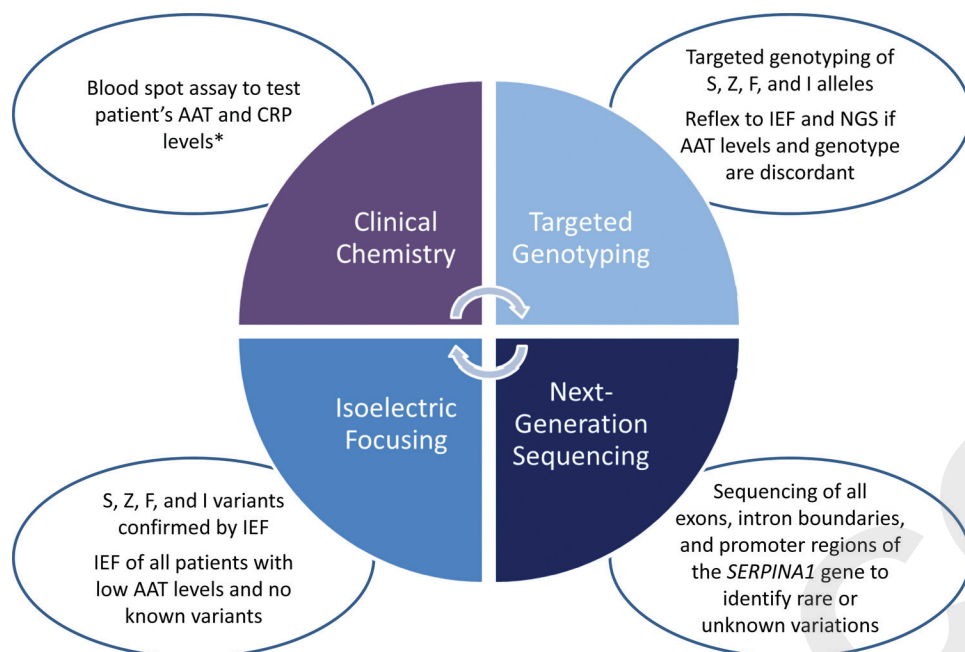


Figure 3. DNA₁ Advanced α -1 Screening Program. The DNA₁ program allows patients with α -1 antitrypsin deficiency (AATD) to be quickly and accurately diagnosed, in particular, those with rare genotypes. The program consists of clinical chemistry (quantitation of both α -1 antitrypsin [AAT] and C-reactive protein levels [CRP]), qualitative assessment of AATD genotypes by targeted genotyping, next-generation sequencing (NGS), and isoelectric focusing (IEF) (in which all loss-of-function variants are identified by a second method to assure accuracy). *CRP measurements are included to identify an acute-phase response, which may mask AATD.

broadly similar.⁴¹ For diagnostic testing of individuals with symptoms of AATD, it is recommended that genotyping for at least the S and Z alleles be undertaken.⁴¹ Advanced or confirmatory testing should include quantitative analysis, phenotyping, and/or expanded genotyping. Once an individual has been identified with an abnormal gene, his or her parents, siblings, and children, as well as extended family, should be provided with the option of genetic counseling and testing for AATD. For family testing, quantitative analysis alone is not recommended because it does not fully characterize the disease risk of AATD.⁴¹

Despite these recommendations, AATD is under-recognized, and there often are significant delays between the onset of symptoms and the initial diagnosis. In the United States, mean diagnostic delays of 5.6 years have been reported.⁴³ The implementation of physician alerts, which recommended testing patients for AATD whose pulmonary function test results showed airflow obstruction, increased the frequency of tests performed.^{44,45} However, they did not improve the rate of diagnosing severe AATD. Therefore, further measures would need to be implemented to enhance the detection of AATD. The combination of a targeted awareness campaign (brochures sent to pulmonary and pediatric specialists) with the offer of free diagnostic screening resulted in the detection of a significant number of individuals with severe AATD.⁴⁶

Although not currently recommended, targeted population screening may be beneficial in populations in which the prevalence of AAT deficiency is high.¹⁷ A nationwide neonatal screening program for AATD was carried out in Sweden. Of 200,000 neonates born between 1972 and 1974, 120 were identified with a PIZ

phenotype.⁴⁷ Furthermore, in a screening study of adult residents of a small Italian alpine village, the prevalence and frequency of all AATD-related alleles, including Z and S, were higher than expected for Italy.⁴⁸ However, these results are unlikely to be applicable to North American populations, due to the higher frequencies of deficiency alleles in the screened populations. In addition, children with AATD will not experience respiratory symptoms until adult life, and only a small percentage may develop childhood liver complications. These reasons, along with issues that relate to the psychosocial effects of diagnosis with a genetic disease on children and their parents, are possible explanations for the limited number of screening programs undertaken. Despite this, a greatly reduced rate of smoking in adulthood was observed in individuals who had been diagnosed with AATD at birth,⁴⁹ which has clear benefits for these patients. Therefore, an expert panel recently recommended that the position on screening programs be reconsidered. This panel suggested a series of pilot studies focused on generating new data on the risks and benefits of population-level newborn screening.⁵⁰

TESTING KITS AND SCREENING PROGRAMS

The key to improving AATD diagnosis rates is the ability to screen individuals who are at risk by using a combination of both qualitative and quantitative testing methods. Used in conjunction, these methods allow the identification of the four different AAT protein categories: normal, deficient, dysfunctional, and null. To accurately diagnose individuals with AATD, test kits usually involve several diagnostic steps to provide

a comprehensive clinical picture and better informed patient management. Single-step test kits, *e.g.*, the AlphaKit QuickScreen (Grifols, Barcelona, Spain), can be used at the point of care. Launched in 2014, this kit requires a few drops of blood and generates results in 15 minutes. However, the kit only detects the Z allele and provides only an initial indication of the presence of AATD. To confirm the diagnosis, further laboratory testing *via* the National Targeted Alpha-1 Detection Program (testing conducted by GeneAidix, LLC, Alachua, FL; sponsored by Grifols) is performed.

GeneAidix also conducts two other α -1 detection programs: the State of Florida Alpha-1 Detection Program, and the Alpha-1 Coded Testing study. The former has been sponsored by the State of Florida Department of Health and Human Services and the Alpha-1 Foundation since 1999. The program was set up as a population screening tool, and testing is free to Florida residents, with finger-stick tests provided by The Alpha-1 Foundation.⁵¹ The Alpha-1 Coded Testing study is currently run by The Medical University of South Carolina, which is conducting a research study about the perceived risks and benefits of genetic testing.⁵¹ Another well-established nationwide testing program, The Alpha₁ Center, Salt Lake City, Utah, uses the AlphaTest Kit (sponsored by Baxalta [Bannockburn, IL; now part of Shire, Dublin, Republic of Ireland]).⁵² To date, the operation has tested >200,000 samples, and diagnostic testing focuses on the most common alleles that make up 95% of *SERPINA1* mutations (S and Z) as well as an expanding panel of rare mutations.⁵²

The most recent screening program to be introduced in the United States is the DNA₁ Advanced α -1 Screening (Biocerna, Gaithersburg, MD). The program has tested samples (collected on serum separator cards) since September 2013. The program seeks to improve the diagnosis of AATD (particularly of rare genotypes) by providing a comprehensive diagnostic workflow, including clinical chemistry (quantitation of both AAT and CRP levels), qualitative assessment of AATD genotype by targeted genotyping, NGS, and IEF (in which all loss-of-function variants are identified by a second method to assure accuracy [Fig. 3]). Initial screening includes genotyping of S, Z, F, and I alleles as well as AAT and CRP levels of all patients. If needed, testing progresses (“reflexes”) onto the next stage, which uses both IEF and NGS. This allows for comprehensive and robust identification of all known, and unknown and/or novel variants of the *SERPINA1* gene, including multiple variants in individuals who might have been previously misdiagnosed.⁵³ By using the above workflow, researchers at Biocerna have found several novel AAT variants that have not been previously described and that were not identifiable by using traditional testing methods.

CONCLUSION

AATD is widely underdiagnosed, and traditional testing practices have limitations. Screening programs that incorporate NGS are the most comprehensive methods available for the accurate diagnosis of AATD, especially for individuals with rare or novel AATD variants. Physicians caring for patients with COPD need to be aware of AATD and the availability of rapid and accurate diagnostic testing. Early diagnosis is key to enabling proactive intervention and to limiting the progression of AATD-related emphysema.

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