Rapid and Inexpensive Detection of α_1 -Antitrypsin Deficiency-Related Alleles S and Z by a Real-Time Polymerase Chain Reaction Suitable for a Large-Scale Population-Based Screening

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α_1 -Antitrypsin (AAT) deficiency is one of the most common genetic disorders in Caucasians, leading to early onset pulmonary emphysema and/or liver disorders. Accumulating data suggest that AAT deficiency is commonly under-recognized or misdiagnosed by physicians. The need for a rapid, timesaving, and relatively inexpensive but reliable detection method for the two most common deficiency alleles was developed using real-time polymerase chain reaction (PCR) genotyping. We designed and validated a 5'-nuclease assay for typing of the PI*S and PI*Z alleles using dual-labeled target-specific fluorescent probes. As a reference method, we used restriction fragment length polymorphism. The real-time PCR method was tested on a large, cross-sectional epidemiological trial. Overall, we genotyped about 1200 samples and found a very good concordance with AAT serum levels and restriction fragment length polymorphism results. In addition, external interlaboratory validation confirmed the accuracy of the real-time PCR method. In our experience, the real-time qualitative PCR using 5'-nuclease assay is suitable as a genetic test for AAT deficiency. This method offers an acceptable balance between reliability and expenses. It seems appropriate for both population-based screening and clinical diagnosis of the deficiency. (*J Mol Diagn* 2007, 9:99–104; DOI: 10.2353/jmoldx.2007.060048)

α_1 -Antitrypsin (AAT) deficiency is one of the most common Mendelian disorders in Caucasians. AAT is a glycoprotein produced mainly in the liver, but its main function is to protect the lung against proteolytic damage from neutrophil elastase. The most common clinical manifestation of AAT deficiency is the early onset panacinar emphysema and chronic obstructive pulmonary disease (COPD). The disease develops at about the age of 45 years,¹ but its onset is accelerated in heavy smokers² and in subjects exposed to severe air pollution.³ Liver

diseases, including hepatitis, cirrhosis, hepatoma, and possibly vasculitis, represent other spectra of clinical manifestations of AAT deficiency.^{4–6}

The protease inhibitor (PI) locus, coding for AAT, is highly polymorphic. Currently, over 100 genetic variants of the sequence have been catalogued. There are only two common variants related to AAT deficiency. The PI*Z allele is characterized by an E342K substitution, caused by GAG to AAG transition in exon 5 and is associated with a severe reduction of serum AAT level. PI*S is identified by an E264V substitution, due to GAA to GTA transversion in the exon 3, and causes a mild reduction in the protein level.⁷

In Europe, frequencies of PI*Z allele range from 0 to 30 per 1000, whereas the prevalence of PI*S allele varies over a wider range from 5 to 150 per 1000.^{8–11} The geographical distribution of these alleles outside Europe depends on colonization and migration vectors. For example, in the United States, Canada, and Australia, the frequencies among Caucasians are similar to those reported in Europe. Both deficiency alleles are rare or absent in African or Asian population.^{12,13} Presently, with the world population at 6.4 billion, 170 million carriers and 4.9 million subjects at risk can be expected.¹⁰ AAT deficiency, although infrequent, is also rarely diagnosed.¹⁴ Moreover, a mean age of the diagnosis is 45 years, whereas an average interval between onset of symptoms and diagnosis is about 8 years. There has not been any progress in early diagnosis of AAT deficiency since the late 1960s.¹⁵

The genetic variability of the PI system was initially studied by a starch gel electrophoresis assay.¹⁶ Currently, diagnosis of AAT deficiency is based on the measurement of AAT levels in the serum and/or isoelectric focusing of the serum within a narrow pH range on the polyacrylamide gel.^{17,18} The latter is the standard for AAT phenotyping. Unfortunately, this technique is time consuming and gives results that are difficult to interpret,

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Table 1. Primers and Probes Sequences for RFLP and Real-Time Qualitative PCR Assay

Name	Length	Sequence*
RFLP		
Primer S forward	23-mer	5'-AAGGTGCCTATGATGAAGCGTTT
Primer S reverse	24-mer	5'-ATGATATCGTGGGTGAGTTCATGT [*]
Primer Z forward	24-mer	5'-CATAAGGCTGTGCTGACCATCGT [~] C
Primer Z reverse	24-mer	5'-TTCCCATGAAGAGGGGAGACTTGG
Real-time PCR		
Primer S forward		The same as for RFLP
Primer S reverse	22-mer	5'-TCAGTCCCAACATGGCTAAGAG
Probe S wild type	22-mer	5'-[ROX]-TGGGTGAGTTCATTTTCCAGGT-[BHQ2]
Probe S mutant	25-mer	5'-[FAM]-ATATCGTGGGTGAGTTCATTTT <u>ACCA</u> -[BHQ1]
Primer Z forward	21-mer	5'-GCTTCCTGGGAGGTGTCCACG
Primer Z reverse		The same as for RFLP
Probe Z wild type	26-mer	5'-[ROX]-CCAGCAGCTTCAGTCCCTTTCTCGTC-[BHQ2]
Probe Z mutant	26-mer	5'-[FAM]-CCAGCAGCTTCAGTCCCTTTCT <u>TGTC</u> -[BHQ1]

*Bold indicates mutagenic nucleotide; caret indicates site of restrictase digestion; underline indicates polymorphic nucleotide. BHQ, Black Hole Quencher.

limiting its availability to a few reference laboratories. Measurement of the AAT level is a simple and widely available test. However, it can be used only as the first step in the evaluation of the deficiency because AAT, as an acute-phase protein, can raise its serum concentration to a normal level during inflammation or trauma.

Introduction of a real-time PCR assay, a novel technique allowing avoidance of postamplification processing, inspired us to design and validate a quick but relatively inexpensive method of genotyping for the two most common AAT deficiency alleles. We chose the most simple chemistries for the qualitative real-time PCR, dual-labeled fluorescent oligoprobes and the 5'-nuclease assay. We expected this method to fit our requirements not only for large-scale population screening but also for rapid clinical diagnosis.

Materials and Methods

Genomic DNA Extraction

Genomic DNA was extracted from blood drawn on EDTA using a simple method of chaotropic lysis of white cells. In brief, 5 ml of blood was mixed with 50 μ l of 6% high-molecular weight dextran (T500; Pharmacia, Peapack, NJ) and left for half an hour at 4°C. Then, the rich cell plasma (1.5 ml) was centrifuged for 10 minutes at 10,000 $\times$ g at room temperature to pellet white blood cells. The pellet was lysed with 400 μ l of DNAzol Reagent (Invitrogen, Carlsbad, CA) and DNA precipitated with 200 μ l of 95% ethanol. Following a wash in 70% ethanol, the DNA precipitate was air-dried, dissolved in 300 μ l of distilled water, and stored at -18°C. Informed consent was obtained from all participants for genetic analysis, and the study was accepted by the University Ethics Committee.

Restriction Fragment Length Polymorphism (RFLP) with Mutagenic Primers

This method was used as the reference to select appropriate genomic templates for optimization of the real-time

PCR assay and to verify the genotyping results. Since the PI*Z allele was not recognized by any available restriction nucleases, one of the primers introduced a restriction site by a base substitution near the PI*Z mutation. A similar design was used for detection of the allele PI*S (Table 1). PCR reactions were performed using the following conditions: total volume of 25 μ l containing 2 μ l of the genomic DNA template, 0.5 U of *Taq* polymerase (Finnzymes, Espoo, Finland), 5 pmol of each primer (TIB MOLBIOL, Berlin, Germany), and 2.5 nmol of each dNTP (Fermentas, Hanover, MD) in a buffer composed of 15 mmol/L ammonium sulfate, 60 mmol/L Tris-HCl, pH 8.9, 3.5 mmol/L magnesium chloride, 0.02% Tween 80, and 0.002% mercaptoethanol. Temperature profiles for PI*Z allele were as follows: initial denaturation at 96°C for 2 minutes; 36 cycles of 96°C for 30 seconds, 68°C for 1 minute; and final extension at 72°C for 10 minutes. PCR conditions for PI*S mutation differed by three-step cycles including denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. PCR was performed on Thermal Cycler T3 (Biometra, Göttingen, Germany). PCR products (4 μ l) were digested with 2 U of *TaqI* (Fermentas) restriction enzyme for detection of the PI*Z mutation. *RsaI* (Fermentas) restriction enzyme was used for genotyping of the PI*S allele. Restriction fragments were stained with YOYO-3 (Molecular Probes, Eugene, OR) intercalating dye at the final concentration of 0.33 μ mol/L and resolved by electrophoresis using a system of automated laser fluorescence in 6% polyacrylamide nondenaturing gel (ALF-express DNA Sequencer; Pharmacia Biotech, Uppsala, Sweden) or a high-resolution agarose gel.

PCR Primers and Probes for the Real-Time Fluorescence Assay

For each assay four oligonucleotides were used: a pair of PCR primers and a pair of dual-labeled allele-specific fluorescent probes. The primers were designed to meet criteria of hybridization at the temperature between 55 and 60°C and the probes to have 5 to 10°C higher annealing temperature than the primers. In addition, gen-

eral guidelines for primer and probe design were followed to avoid primer-dimers or sequence self-complementary artifacts.

The two primer sets amplified a 248-bp sequence spanning the region of the PI*Z mutation site and a 260-bp sequence flanking the PI*S mutation site. One primer in the each set was the same as used for RFLP (Table 1).

Two sets of probes, complementary to the flanking sequence of the mutations, were labeled at the 5' end with the reporter dye and at the 3' end with the quencher (Black Hole Quencher). For PI*Z reaction, the probes differed only by the one variable nucleotide. The probe for a wild-type sequence in the PI*S allele reaction was additionally shifted by three nucleotides upstream and shortened by three bases to improve the specificity of the assay (see Results). The variable nucleotides for each of the sets of probes were near the 3' ends. Wild-type probes were labeled with 6-carboxy-X-rhodamine (ROX), and the ones for the mutant alleles with 6-carboxyfluorescein (FAM) (Table 1).

PCR Conditions for Real-Time Fluorescence Assay

The real-time allelic discrimination assay, based on 5'-nuclease activity of *Taq* polymerase, was performed in a total volume of 50 μ l containing 3 μ l of the genomic DNA template, 1 U of *Taq* polymerase (Finnzymes), 20 pmol of each primer (TIB MOLBIOL), 2 pmol of each probe (IDT DNA, Coralville, IA), 10 nmol of each dNTP (Fermentas), and 2% dimethyl sulfoxide (Sigma, St. Louis, MO). The reaction buffer was the same as for PCR-RFLP. Amplification was run on the thermocycler with optical module (iCycler iQ; Bio-Rad, Hercules, CA). Cycling protocols were as follows: for PI*S mutation, initial denaturation at 96°C for 2 minutes, followed by 40 cycles of denaturation at 96°C for 30 seconds, and annealing/extension at 61.7°C for 90 seconds; for PI*Z detection, these parameters differed only in the temperature of annealing/extension step, which was 68.8°C. Fluorescence data were collected for each cycle by the end of annealing/extension using appropriate filter sets: excitation/emission for FAM 490/530 nm and for ROX 575/620 nm. Experiments were conducted in a 96-well plate format covered with optically clear sealing tape. Each plate contained also three replicates of positive controls: wild type, mutant hetero-, and homozygote, and two blanks with no DNA template added. The genotyping results were analyzed using iCycler iQ Optical System Software v.3 (Bio-Rad). During the combined annealing/extension step of the amplification, once the complementary probe hybridized to the target, polymerase cleaved off the reporter nucleotide, unmasking the fluorescence signal. The fluorescence increased with cycling in parallel to the accumulating product of the specific allele.

Figure 1. Ethidium bromide-stained agarose gel showing results of amplification and digestion in RFLP method (DNA size marker, pBR322 DNA/*Aha*I). MM samples should be interpreted as non-PI*S and non-PI*Z.

Results

RFLP was used as a comparative method for the real-time PCR assay. The nucleotides generating restriction sites were located at one base before the 3' end of the primers. In the PI*S allele reaction, the *Rsa*I restriction site was introduced only in the presence of mutation, and the product was digested into fragments of 143 and 24 bp. The wild-type allele was not cleaved (167 bp). In the PI*Z allele digestion, the wild-type sequence was cut with *Taq*I at 137 and 23 bp, but there was no cleavage of PCR product in the presence of PI*Z mutation (160 bp). Results of the conventional agarose gel electrophoresis and the automated laser fluorescence electrophoresis are presented in Figures 1 and 2. The RFLP method was validated using external reference samples of DNA with known deficiency genotypes.

Typical results of the real-time fluorescent reactions are shown in Figure 3. At each cycle, an increase in the 5' dye fluorescence signal corresponded to accumulation of the specific amplification product. Traces of ROX signal for most samples demonstrated an increase in fluorescence (Figure 3A) because probes for

Figure 2. Automated laser fluorescence electrophoresis. First trace, DNA size marker (pBR322 DNA/*Aha*I); next five traces, RFLP assay for PI*S mutation detection; and the last five, for PI*Z allele. In the case of PI*Z heterozygotes, some inequality of the peaks is seen, not interfering with the interpretation of results. MM samples should be interpreted as non-PI*S and non-PI*Z.

Figure 3. Real-time PCR with dual-labeled fluorescent probes. PCR amplification plots were baseline-subtracted and fitted automatically by the software. Increase in fluorescence is expressed in relative fluorescence units (RFU). ROX dye labeled the probe for a wild-type allele (A); FAM marked the probe for a mutant allele (B). The genotypes were automatically scored using allelic discrimination graph for ROX versus FAM (C). See Results for details.

the non-PI*S or non-PI*Z alleles bound to the template, and reporter nucleotide was cleaved off. Three samples at this plot did not have any increase in ROX fluorescence, corresponding to non-template samples and mutant homozygote as the positive control. A separate group of nine samples was heterozygotes and showed a diminished rise in the ROX fluorescence. Corresponding traces of FAM fluorescence (Figure 3B) reflected the presence of the mutated allele, thus complementing the assay. In most samples, only an unspecific increase in the FAM fluorescence was observed during terminal cycles. In 10 samples, a high or moderate increase of FAM signal reflected one mutated homozygote and nine heterozygotes. In our protocol, the probe for wild-type allele was more specific, whereas the one for mutated target was more sensitive. Genotype assignment was performed using a com-

bined graph of ROX versus FAM signals (Figure 3C). The most numerous clusters consisted of samples with high ROX signals representing wild-type homozygotes. A high FAM signal value was characteristic for the mutated homozygote. Samples with moderate FAM and ROX signals were typically observed in heterozygotes. A very low fluorescence for both reporters was obtained with non-template controls. The method repeatedly produced easy to interpret sample clusters.

In addition to a fine adjustment for annealing/extension temperature, the real-time PCR assay required a redesign of one probe. Initially, the probe for PI*S allele differed only in the one variable nucleotide from the wild-type allele. However, the wild-type probe demonstrated thermal instability, manifested by an unspecific increase in ROX fluorescence during the first cycles of amplification. This phenomenon was independent of the addition of *Taq* polymerase. Although this probe retained some specificity, amplitude of the signal was very weak. A shift of the probe position in relation to the variable nucleotide with subsequent adjustment of the length to match melting temperature was sufficient to obtain a reliable signal. Thus, in our hands, a set of probes differing in more than one nucleotide showed a satisfactory allelic discrimination.

Template DNA concentration, as verified by spectrophotometry, was in a wide range from 5 to 400 ng/ μ l. This did not create any problem during amplification procedure or influence reliability of the results. Water solution of genomic DNA was stable for a period of several months; in addition, samples stored over 5 years still gave clear results of real-time PCR genotyping.

The validation of this approach consisted of comparison of AAT genotypes assigned by the two different methods in a sample of 550 subjects from a population trial. As a result, 12 heterozygotes MZ, 17 heterozygotes MS, and one homozygote S were found. During the validation phase, every mutated genotype and 202 randomly selected non-PI*S/non-PI*Z genotypes were analyzed with the RFLP method for PI*S (71 samples) and for PI*Z (131 samples). Validation established 100% agreement between the two methods. No case of miscalling the alleles was encountered. Genotyping failure for the real-time PCR method was 4.2% and was exclusively due to a very low concentration of DNA template, which limited fluorescence amplitude. The same samples were genotyped with 1.3% amplification failure by the RFLP method. In both methods, the problem was corrected by an increase of genomic DNA template.

In the population trial, samples of serum were obtained from all participants ($n = 550$), and AAT concentration was measured using laser immunonephelometry (Dade Behring, Marburg, Germany), with a normal range of 0.9 to 2.0 g/l. Subjects with the wild-type genotype had a mean AAT level of 1.26 g/l (range: 0.85 to 2.51), MZ heterozygotes averaged 0.76 g/l (range: 0.66 to 0.85), and MS heterozygotes, 1.09 g/l (range: 0.84 to 1.33). Measurements of AAT should only be the first step in diagnosis of deficiency, because heterozygous genotypes hardly can be distinguished by the level of total serum AAT.¹⁹

The real-time PCR genotyping method was subsequently used to study an extended sample of population ($n = 460$), 50 patients with pulmonary symptoms and depressed serum level of AAT. Next, the members of two families identified by PI*ZZ homozygotes were investigated. In this group, four PI*ZZ homozygotes and seven heterozygotes were detected. Familial segregation of the deficiency allele was confirmed. The method was also successful in PI*S and PI*Z typing of DNA extracted from 100 fresh-frozen biopsies.

Overall, we genotyped over 1200 individuals using the real-time PCR method. Eventually, after validation and external quality control (Deutsche Vereinigte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin, Referenzinstitut für Bioanalytik, Bonn, Germany), this method will be introduced to common clinical practice in our hospital and the region of Southern Poland.

Discussion

When we designed our RFLP assay, there were no available restriction enzymes that recognized the PI*S and PI*Z mutation. Thus, the most popular method of typing the alleles was PCR-mediated site-direct mutagenesis.^{20–23} Currently, there are specific restriction endonucleases available, and RFLP assay can be performed in a multiplex approach.²⁴ However, this method remains time consuming because it requires post-PCR product digestion and electrophoresis, making it difficult for the genotyping of large population groups. In our study, the RFLP method was used only as a reference test for the real-time PCR. Likewise, in two previous reports,^{25,26} RFLP was chosen as a reference method, instead of direct sequencing. We redesigned our PCR-RFLP to simplify and shorten the time of electrophoresis and facilitate interpretation with automated polyacrylamide gel separation.

Real-time PCR seems the method of choice for genotyping the two most common AAT deficiency alleles in population trials. Considering costs of reagents and labor, it is relatively inexpensive and timesaving. This technique was used to detect AAT deficiency for the first time in the late 1990s,^{25,27} also in a multiplex reaction.²⁶ The earlier methods of genotyping relied on hybridization probes and melting-curve analysis. This was performed on LightCycler (Roche Diagnostics, Mannheim, Germany) in a glass capillaries format.^{25–27} Recently, novel real-time PCR probes were developed, eg, molecular beacons and scorpions, which could be used for detection of other deficiency alleles.²⁸

To our knowledge, this is one of the first reports on real-time qualitative PCR using a simple dual-labeled oligoprobe and 5'-nuclease assay in the molecular detection of AAT deficiency. Behrens and Lang reported on genetic tests for AAT deficiency using the TaqMan technology, but they did not include sequences of probes as commercial kits were used.²⁹ An advantage of this method could be a more reliable result in case of another mutation near the probe target

site. In such an instance, mismatch other than that expected would change a melting curve characteristic. An abnormal melting point could hardly distinguish the deficiency allele from other genetic variation. Using 5'-nuclease assay, any other variance of the sequence should impair efficacy of hybridization to the degree preventing any signal of the other variant allele. Due to a highly polymorphic sequence of AAT locus in the neighborhood of PI*Z mutation, a risk of false results of the 5'-nuclease assay might be encountered with the synonymous G to A transition in the lysine 343 codon (rs1050520) or the synonymous G to C transversion in 349 threonine codon (rs1050469). However, both sequence variants seem rare, and their population frequencies were not determined. In our experience, 5'-nuclease assay was the most simple, inexpensive, reliable, and robust method of AAT genotyping. Despite quite a demanding optimization of parameters, the reaction once set was highly reproducible and did not require further modifications. Thus, it was a golden mean between the more advanced real-time PCR techniques and costs or accessibility. Due to a moderate technical requirement, the method could be run on an average thermocycler with the optical module.

We report on a detection method tested on a much larger number of samples than in the previous publications.^{25–27} In our opinion, the assay passed validation using the reference test and the interlaboratory control. It warrants not only application in research but also in population screening and/or clinical diagnosis. The limitation of the method is the same as of the genotyping in opposition to the phenotyping. It detects only two deficiency alleles, PI*S and PI*Z; thus, other genotypes should be correctly named as non-PI*S and non-PI*Z. Alleles PI*S and PI*Z are the most commonly mutated ones, comprising over 95% of deficiency cases. However, if genotyping resulted in non-PI*S and non-PI*Z in case of decreased AAT serum level, the next diagnostic step would require phenotyping.

In our study, we used DNA samples extracted from whole blood. A truly rapid screening test should work on more easily accessible DNA template, eg, from mouthwash or a dried blood spot. This could be the next step in testing of the presented method. As it worked with a genomic DNA template in concentration exceeding 20 ng/ μ l, an optimized and simple DNA extraction should easily meet this criterion.

The American Thoracic Society/European Respiratory Society¹⁹ recently published standards for the diagnosis of individuals with AAT deficiency. These recommendations of diagnostic tests vary on specific situations, including a prevalence of AAT deficiency in the population. One of the most important issues in organization of a national service for individuals with AAT deficiency is a reliable prediction of the frequency based on a representative population sample. Although our method was designed and validated for that purpose, it turned out to be useful also in clinical diagnostics.

References

- McElvaney NG, Stoller JK, Buist AS, Prakash UB, Brantly ML, Schluchter MD, Crystal RD: Baseline characteristics of enrollees in the National Heart, Lung and Blood Institute Registry of alpha 1-antitrypsin deficiency. Alpha 1-Antitrypsin Deficiency Registry Study Group. *Chest* 1997, 111:394–403
- Piitulainen E, Eriksson S: Decline in FEV1 related to smoking status in individuals with severe alpha1-antitrypsin deficiency (PiZZ). *Eur Respir J* 1999, 13:247–251
- Szczeklik A, Stankowska K, Frydecka I: Cardiopulmonary function in 1-antitrypsin heterozygotes exposed to severe air pollution. *Am Rev Respir Dis* 1973, 107:289–291
- Carrell RW, Lomas DA: Alpha1-antitrypsin deficiency—a model for conformational diseases. *N Engl J Med* 2002, 346:45–53
- Needham M, Stockley RA: Alpha 1-antitrypsin deficiency. 3: Clinical manifestations and natural history. *Thorax* 2004, 59:441–445
- Stoller JK: Alpha1-antitrypsin deficiency. *Thorax* 2004, 59:92–93
- DeMeo DL, Silverman EK: Alpha1-antitrypsin deficiency. 2: Genetic aspects of alpha(1)-antitrypsin deficiency: phenotypes and genetic modifiers of emphysema risk. *Thorax* 2004, 59:259–264
- Blanco I, Fernandez E, Bustillo EF: Alpha-1-antitrypsin PI phenotypes S and Z in Europe: an analysis of the published surveys. *Clin Genet* 2001, 60:31–41
- Blanco I, de Serres FJ, Fernandez-Bustillo E, Lara B, Miravittles M: Estimated numbers and prevalence of Pi*S and Pi*Z alleles of alpha1-antitrypsin deficiency in European countries. *Eur Respir J* 2006, 27:77–84
- de Serres FJ: Worldwide racial and ethnic distribution of alpha1-antitrypsin deficiency: summary of an analysis of published genetic epidemiologic surveys. *Chest* 2002, 122:1818–1829
- Hutchison DC: Alpha 1-antitrypsin deficiency in Europe: geographical distribution of Pi types S and Z. *Respir Med* 1998, 92:367–377
- Blanco I, Bustillo EF, Rodriguez MC: Distribution of alpha1-antitrypsin PI S and PI Z frequencies in countries outside Europe: a meta-analysis. *Clin Genet* 2001, 60:431–441
- de Serres FJ, Blanco I, Fernandez-Bustillo E: Genetic epidemiology of alpha-1 antitrypsin deficiency in North America and Australia/New Zealand: Australia, Canada, New Zealand and the United States of America. *Clin Genet* 2003, 64:382–397
- de Serres FJ: Alpha-1 antitrypsin deficiency is not a rare disease but a disease that is rarely diagnosed. *Environ Health Perspect* 2003, 111:1851–1854
- Campos MA, Wanner A, Zhang G, Sandhaus RA: Trends in the diagnosis of symptomatic patients with alpha1-antitrypsin deficiency between 1968 and 2003. *Chest* 2005, 128:1179–1186
- Fagerhol MK, Laurell CB: The polymorphism of "prealbumins" and alpha-1-antitrypsin in human sera. *Clin Chim Acta* 1967, 16:199–203
- Allen RC, Harley RA, Talamo RC: A new method for determination of alpha-1-antitrypsin phenotypes using isoelectric focusing on polyacrylamide gel slabs. *Am J Clin Pathol* 1974, 62:732–739
- Massi G, Fabiano A, Ragusa D, Auconi P: Characterization of alpha-1-antitrypsin by isoelectric focusing on an ultrathin polyacrylamide gel layer. An economic high-resolution system for determining PiM subtypes. *Hum Genet* 1979, 53:91–95
- American Thoracic Society/European Respiratory Society Statement: Standards for the diagnosis and management of individuals with alpha-1 antitrypsin deficiency. *Am J Respir Crit Care Med* 2003, 168:818–900
- Andolfatto S, Namour F, Garnier AL, Chabot F, Gueant JL, Aimone-Gastin I: Genomic DNA extraction from small amounts of serum to be used for alpha1-antitrypsin genotype analysis. *Eur Respir J* 2003, 21:215–219
- Braun A, Meyer P, Cleve H, Roscher AA: Rapid and simple diagnosis of the two common alpha 1-proteinase inhibitor deficiency alleles Pi*Z and Pi*S by DNA analysis. *Eur J Clin Chem Clin Biochem* 1996, 34:761–764
- Lam CW, Pang CP, Poon PM, Yin CH, Bharathi G: Rapid screening for alpha1-antitrypsin Z and S mutations. *Clin Chem* 1997, 43:403–404
- Tazelaar JP, Friedman KJ, Kline RS, Guthrie ML, Farber RA: Detection of alpha 1-antitrypsin Z and S mutations by polymerase chain reaction-mediated site-directed mutagenesis. *Clin Chem* 1992, 38:1486–1488
- Ferrarotti I, Zorzetto M, Scabini R, Mazzola P, Campo I, Luisetti M: A novel method for rapid genotypic identification of alpha 1-antitrypsin variants. *Diagn Mol Pathol* 2004, 13:160–163
- Aslanidis C, Nauck M, Schmitz G: High-speed detection of the two common alpha(1)-antitrypsin deficiency alleles Pi*Z and Pi*S by real-time fluorescence PCR and melting curves. *Clin Chem* 1999, 45:1872–1875
- von Ahsen N, Oellerich M, Schutz E: Use of two reporter dyes without interference in a single-tube rapid-cycle PCR: alpha(1)-antitrypsin genotyping by multiplex real-time fluorescence PCR with the Light-Cycler. *Clin Chem* 2000, 46:156–161
- Ortiz-Pallardó ME, Zhou H, Fischer HP, Neuhaus T, Sachinidis A, Vetter H, Brüning T, Ko Y: Rapid analysis of alpha1-antitrypsin PiZ genotype by a real-time PCR approach. *J Mol Med* 2000, 78:212–216
- Orrù G, Faa G, Pillai S, Pilloni L, Montaldo C, Pusceddu G, Piras V, Coni P: Rapid PCR real-time genotyping of M-Malton alpha1-antitrypsin deficiency alleles by molecular beacons. *Diagn Mol Pathol* 2005, 14:237–242
- Behrens M, Lange A: A highly reproducible and economically competitive SNP analysis of several well characterized human mutations. *Clin Lab* 2004, 50:305–316