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# Immunonephelometry and Reverse Hybrydization Genotyping in Diagnosis of Alpha-1-Antitrypsin Deficiency in Macedonians

Olivija Efinska-Mladenovska, Dejan Trajkov, Aleksandar Petlichkovski, Olgica Sibinovska, Slavica Hristomanova Mitkovska, Mirko Spiroski*

*Institute of Immunobiology and Human Genetics, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia*

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**Key words:** alpha-1-antitrypsin deficiency; AAT serum concentration; SERPINA1 gene; nephelometry; reverse line strip hybridization; Republic of Macedonia.

***Correspondence:** Prof. Dr. Mirko Spiroski. Institute of Immunobiology and Human Genetics, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, 1109 Skopje, Republic of Macedonia. E-mail: mspiroski@yahoo.com

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**Abstract**

**BACKGROUND:** With a frequency of 1:1600, the alpha-1-antitrypsin deficiency is one of the most frequent hereditary diseases and can be recessively inherited. AAT deficiency is most often caused by inheritance of the so-called PiZ allele. Inheritance of this allele increases the risk of developing chronic obstructive pulmonary diseases (COPD) and liver disease.

**AIM:** The aim of this study was to present immunonephelometry and reverse hybridization genotyping in diagnosis of alpha-1-antitrypsin deficiency in Republic of Macedonia.

**MATERIAL AND METHODS:** At the Institute of Immunobiology and Human Genetics, part of the Faculty of Medicine in Skopje, in the previous 7 years, total of 361 patients with suspected alpha-1-antitrypsin (AAT) deficiency were referred for analysis of AAT concentration using nephelometry (Dade Behring) and subsequent AAT genotyping of individuals with alpha-1-antytripsin deficiency at protein level, based on reverse hybridization technique.

**RESULTS:** Measurement of AAT concentration (g/l) by nephelometry have shown normal level in the range of 1.37-1.41 g/l (88%), lower than normal AAT levels in the range of 0.70-0.83 g/l (8.03%), and concentration above the normal levels in the range of 2.28-2.4 g/l (3.88%).

**CONCLUSION:** Diagnosis in the case of a suspicion of AAT deficiency is carried out by measuring the alpha-1-antitrypsin level in blood and by genotyping of alpha-1-antytripsin allele.

## Introduction

Alpha-1-antitrypsin (AAT) is a member of the serine protease inhibitor family (Pi), deficiency of which is associated with chronic pulmonary disease (emphysema) and liver disease [1, 2]. AAT is produced in the liver, released into the circulation and enters the lungs by diffusion, where it inhibits neutrophil elastase [3]. AAT is a 394 amino acid, 52 kDa acute phase glycoprotein encoded on the long distal arm of the chromosome 14 (14q32.1) [4].

With a frequency of 1:1600, the alpha-1-antitrypsin deficiency is one of the most frequent hereditary diseases and can be recessively inherited. AAT deficiency is most often caused by inheritance of the so-called PiZ allele. Inheritance of this allele increases the risk of developing chronic obstructive pulmonary diseases (COPD) and liver disease. Little is known about the pathogenesis, as not all people affected actually fall ill. The examination of twins shows that exogenic factors affect the course of disease [5, 6].

The most cases of alpha-1-antitrypsin deficiency are caused by homozygosis for the deficient allele PiZ or by heterozygosis combination of the 2 most common deficient alleles, PiS and PiZ. In the present of Z allele, however, further decreases AAT concentrations occur and may lead to uncontrolled elastase activity and proteolytic damage in the lower respiratory tract [7, 8], increasing the risk for developing COPD. The S allele is a deficiency allele attributable to intracellular degradation [9, 10]. Patients homozygous for the S allele have AAT

concentration ~10%-20% below those typically observed in individuals homozygous for nondeficiency allele. Around 25% of all children with a complete alpha-1-antitrypsin deficiency (homozygous PiZ mutation) develop liver cirrhosis, and approximately 75% of the affected adults suffer from chronic obstructive pulmonary diseases [11, 12].

PiZ is by far the most common and significant deficiency allele with great clinical importance. The PiS allele, also frequent, seems only to be relevant in combination with PiZ, as exclusively PiS homozygos patients are not usually affected. Heterozygous PiMZ and PiSZ carriers are usually clinically inconspicuous or suffer only slightly, unless they smoke. Heterozygous carriers who smoke develop in the majority of cases chronically obstructive pulmonary diseases, comparable to those of clinically homozygous non-smoking carriers. Personal smoking has the most dramatic effect on health status of PiZ/Z individuals. PiZ/Z individuals who smoke cigarettes die 20 years earlier than PiZ/Z nonsmokers [13]. Environmental risk factors for progression of lung disease among individuals with a Z allele include personal smoking, passive smoke exposure especially as a child and mineral dust exposure [14-17].

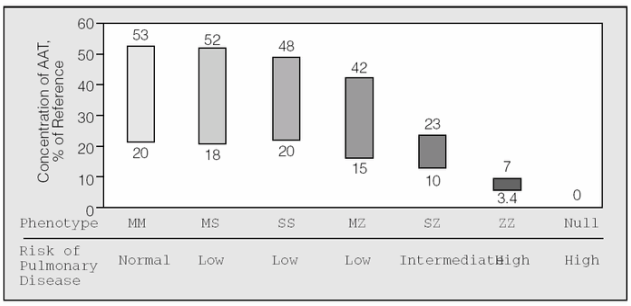


*Figure 1: Serum concentration of AAT according PI genotype. On the X axis, the main PI genotypes are given, and on the Y axis the corresponding plasma concentration of AAT are shown as a proportion of the normal range. The risk of pulmonary disease increases with the ratio dropping below 35%.*

A common AAT classification divides the Pi variants into 4 categories:

1. Normal variants, characterized by normal plasma concentrations of AAT, for which there is no increased risk of developing lung or liver disease. This category includes the M genotypic variant and sub variants.

2. Deficient variants, characterized by lower then normal but detectable concentrations of AAT. This group mainly includes the PiS and PiZ variants. The PiZ variant associated with increased risk of lung or liver disease because it expresses approximately 10% to 20% AAT. The PiS variant, which is more common in the Mediterranean region, expresses approximately 50% to 60% AAT.

3. Null variants, associated with undetectable concentrations of AAT in plasma and with highest risk of emphysema.

4. Dysfunctional variants, characterized with normal amount of AAT, which does not function properly.

The protein level in serum and the enzyme activity depend on the genotype and are distributed in a broad range from 0 to 100% [18] (Figure 1).

The aim of this study was to present immunonephelometry and reverse hybrydization genotyping in diagnosis of alpha-1-antitrypsin deficiency in Republic of Macedonia.

## Material and Methods

At Institute of Immunobiology and Human Genetics, part of the Faculty of Medicine in Skopje, in the previous 7 years, total of 361 patients with suspected alpha-1-antitrypsin (AAT) deficiency were referred for analysis of AAT concentration using neohelometry (Dade Behring) and subsequent AAT genotyping of individuals with alpha-1-antytripsin deficiency at protein level, based on reverse hybridization technique. Samples of whole blood (for AAT genotyping) and serum (for measurement of AAT concentration) were collected for each patient.

Six millilitres of blood were drawn from the patient and his first degree-relatives, by venepunction, after signing a written consent. Genomic DNA was isolated from the peripheral blood leukocytes using phenol-chlorophorm extraction method [19]. The samples of DNA were subsequently stored in the Macedonian Human DNA Bank [20]. The detection of the alpha-1-antytripsin deficiency alleles PiS and PiZ was performed using the commercial Alpha-1-antytripsin kit RDB2010E (GenID GmbH, Straβberg, Germany), based on reverse hybridization technique [21, 22].

Using the kit, alpha-1-antytripsin deficiency alleles PiS and PiZ were detected, by means of a total of 8 DNA probes. The test shows the presence or absence of mutations which encode the exchange of amino acids from glutamic acid to lysine at codon 342 (PiZ), and from glutamic acid to valine at codon 264 (PiS). The GenID alpha-1-antytripsin test enables a reliable detection and differentiation of both homozygous and heterozygous characteristics. It is based on the polymerase chain reaction (PSR) method with hybridization subsequently [21].

A PCR is first carried out using isolated DNA. In this multiplex reaction two fragments of the Alpha-1-antytripsin gene are amplified with specific, biotin-labeled primers. The characterization of the amplified gene fragments takes place in a hybridizing reaction with sequence-specific oligonucleotide probes (SSOP), which were immobilized on nitrocellulose

strip (reverse hybridization). Immobilized probes are specific for the PiM allele (wild type), PiZ (mutation), the wild type and mutated sequence of the S-locus, as well as various control zones.

During hybridization, the denatured amplified DNA binds to the gene probes attached to the strips. A highly specific washing procedure ensures that the hybrids only survive if the probe's sequence is 100% complementary to that of the amplified DNA. Streptavidin-coupled alkaline phosphatase binds to the hybrids of gene probe and biotin-labeled amplified DNA. This complex then was detected by a colour reaction of BCIP/NBT at the alkaline phosphatase. The band pattern was analyzed using the template supplied. A total of eight reaction zones were defined and capable of development (Figure 2).

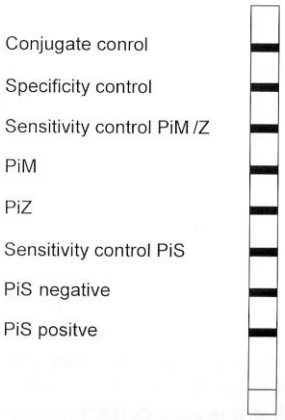


*Figure 2: Control zones and gene probes on the nitrocellulose strip.*

Conjugate control, Sensitivity control PiM/Z and Sensitivity control PiS are reactions zones who must always be developed. The first of them documents the efficiency of the conjugate bonding. The second and third reactions zones document the optimal sensitivity of the hybridization and function as an amplification control. Specificity control is reaction zone which is only developed if the washing temperature is too low. It indicates an unspecific hybridization. PiM (wild type) and PiZ (mutation) are reaction zones which are developed if the wild type sequence PiM apropos mutated sequence PiZ is present. If the patient has heterozygous characteristics, the reaction zone ,,PiM" is also developed. PiS negative (wild type) and PiS positive (mutation) are reaction zones which are developed if the wild type PiS sequence apropos mutated PiS sequence is present. If the patient has heterozygous characteristics, the reaction zone ,,PiS negative" is also developed. A strongly reduced or non-existent concentration of the protein points to a homozygous defect. The AAT concentration in heterozygous patients is usually in the lower normal region. Since the measurement of the alpha-1-antytripsin level in blood is not appropriate for identifying heterozygous carriers, certain diagnosis can only be achieved by typing the alpha-1-antytripsin allele [22].

For measurement on alpha-1-antytripsin level in blood we used nephelometry on a Behring Nephelometer (BN ProSpec) with a commercially available standard and monospecific antisera (Dade Behring), according to the manufacturer s instructions. Nephelometry is the most commonly used measurement principle for the immunochemical determination of protein in serum, urine and other body fluids. It is also the most accurate one. In this method, the light which is scattered by the antigen-antibody complexes is measured. If a sample containing antigen and the corresponding antiserum are put into a cuvette, antigen-antibody complexes are formed. A light beam is generated by means of a laser diode, and this is then sent through the cuvette. The light is scattered by the antigen-antibody complexes which are present. The intensity distribution of the scattered light depends on the relationship of the particle size of the antigen-antibody complexes to the wavelength. Heidelberger-Kendall curve shows the relationship between the antigen level and the measurement signal at a constant antibody level. In the evaluation on the Heidelberger-Kendall curve, two antigen concentrations could therefore be responsible for a particular measurement signal:

- The first point lies on the ascending branch in the antibody excess range (low antigen concentration).
- The second point lies on the descending branch in the antigen excess range (high antigen concentration) (Figure 3).

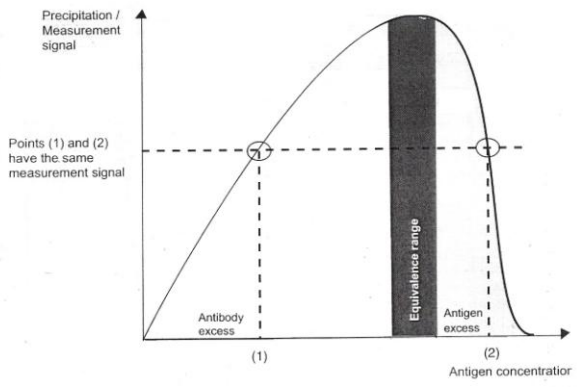


*Figure 3: Heidelberger-Kendall curve.*

## Results

Measurement of AAT concentration (g/l) by nephelometry have shown normal level in the range of 1.37-1.41 g/l (88%), lower than normal AAT levels in

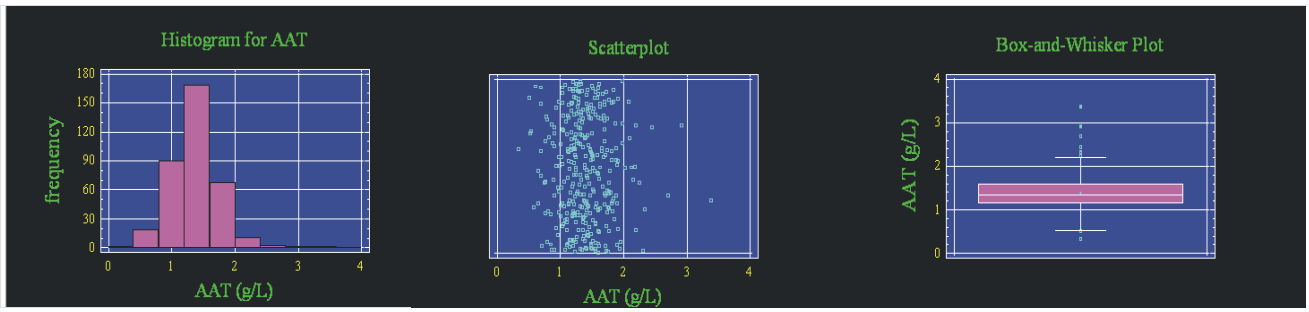


*Figure 4: Frequency distribution of AAT serum concentration of 361 samples, analyzed by immunonephelometry*.

the range of 0.70-0.83 g/l (8.03%), and concentration above the normal levels was seen in the range of 2.28-2.4 g/l (3.88%). Measurement of AAT concentration (g/l) by nephelometry have shown normal level in the range of 1.37-1.41 g/l (88%), lower than normal AAT levels in the range of 0.70-0.83 g/l (8.03%), and concentration above the normal levels was seen in the range of 2.28-2.4 g/l (3.88%) (Figure 4). The genotype assay was interpreted with respect to the PiM, PiZ and PiS alleles. Results of measured AAT concentration by nephelometry and AAT genotyping with reverse hybridization technique are showed in the Table 1.

**Table 1: Results of samples with measured AAT concentration (g/l) and their AAT genotyping.**

| Sample ID | AAT concentration, g/l | Genotype Results |
|---|---|---|
| AT001 | 0.50 | PiZ/Z//PiSN |
| AAT004 | 0.70 | PiM/Z |
| AAT005 | 0.89 | PiM/Z; PiSN/P |
| AAT006 | 1.13 | PiM/M; PiSN |
| AAT007 | 0.88 | PiM/M; PiSN |
| AAT008 | 1.19 | PiM/Z; PiSN/P |
| AAT009 | 1.50 | PiM/Z; PiSN/P |
| AAT010 | 0.61 | PiM/Z; PiSN/P |
| AAT011 | 0.70 | PiM/Z; PiSN/P |
| AAT014 | 1.19 | PiM/Z; PiSN/P |
| AAT017 | 0.51 | PiM/Z; PiSN/P |

An individual with a PiZ/Z genotype is homozygous for the PiZ variation and homozygous for the wild-type allele at the codon associated with the PiS alleles (PiS/N-,,PiS negative"). A similar interpretation is made for an individual with an PiS genotype (PiS/P-,,PiS positive"). If neither the PiZ nor PiS/P allele is detected, it is likely that the individual possesses 2 wild-type alleles PiM/M and PiS/N. If one PiZ and PiM allele is detected, the individual is interpreted as having a PiM/Z genotype, otherwise referred to as a PiZ heterozygote. This individual is heterozygous for the PiZ variation and homozygous for the wild-type allele at the codon associated with the PiS alleles (PiS/N-,,PiS negative"). A similar interpretation is made for an individual in whom two PiS alleles is detected (PiS/N/P).

## Discussion

In this paper we presented our results from immunonephelometry and reverse hybrydization genotyping in diagnosis of alpha-1-antitrypsin deficiency in Republic of Macedonia. Measurement of AAT concentration (g/l) by nephelometry have shown normal level in 88%, lower than normal AAT levels in 8.03%, and concentration above the normal levels was seen in 3.88%. We described for the first time in Republic of Macedonia parallel investigation of AAT concentration in serum and genotypes of SERPINA1 gene.

The SERPINA1 gene (*107400) encodes alpha-1-antitrypsin (AAT), also known as protease inhibitor (PI), a major plasma serine protease inhibitor. AAT complexes predominantly with elastase, but also with trypsin, chymotrypsin, thrombin, and bacterial proteases. The most important inhibitory action of AAT is that against neutrophil elastase (ELANE, or HLE; 130130), a protease that degrades elastin of the alveolar walls as well as other structural proteins of a variety of tissues (review by Cox, 2001) [23]. Long et al., 1984 [24] found that the genomic length of the PI gene is 10.2 kb with a 1,434-bp coding region. The gene has 4 introns; exon 1, the 5-prime portion of exon 2, and the 3-prime portion of exon 5 are noncoding regions. The first intron, 5.3 kb long, contains a 143-amino acid open reading frame (which does not appear to be an actual protein coding region), an Alu family sequence, and a pseudotranscription initiation region.

At the moment there are 40 allelic variants of SERPINA1 gene (Table 2). We investigated three allelic variants (PiM, PiZ and PiS alleles). PI Z allele (.0011, PI, GLU342LYS ON M1A [dbSNP:rs28929474]) is the most frequent allele leading to a high risk of emphysema (and liver disease) in the homozygote; the allele frequency is 0.01-0.02 in US Caucasians (Crystal, 1989) [25]. Nukiwa et al., 1986 [26] demonstrated the val213-to-ala substitution (here symbolized M1A) in PI*Z in addition to the disease-producing glu342-to-lys mutation. Ala213 was found in all of 40 Z haplotypes, using synthetic oligonucleotide gene probes directed toward the mutated exon 3 sequences in the Z gene. PI M(MALTON) (.0012 PI, PHE52DEL ON M2) as liver disease, as well as emphysema, has been described in patients with the rare PI*M(Malton) allele.

**Table 2: Current Allelic Variants of Serpin Peptidase Inhibitor, Clade A, Member 1; SERPINA1 (OMIM 107400).**

| Number ▲ | Phenotype | Mutation | dbSNP |
|---|---|---|---|
| .0001 | PI M1-ALA213 PI, M1A | PI, ALA213 | [rs6647] |
| .0002 | PI M1-VAL213 PI, M1V | PI, ALA213 | [rs6647] |
| .0003 | PI M2 | PI, ARG101HIS ON M3 | [rs709932] |
| .0004 | PI M3 | PI, GLU376ASP ON M1V | [rs1303] |
| .0005 | PI M4 | PI, ARG101HIS ON M1V | - |
| .0006 | PI B(ALHAMBRA) | PI, ASP-LYS | - |
| .0007 | PI F | PI, ARG223CYS ON M1V | [rs28929470] |
| .0008 | PI P(ST. ALBANS) | PI, ASP341ASN ON M1V | [rs28929471], [rs143370956] |
| .0009 | PI X | PI, GLU204LYS ON M1V | [rs199422208] |
| .0010 | PI CHRISTCHURCH | PI, GLU363LYS | [rs121912712] |
| .0011 | PI Z | PI, GLU342LYS ON M1A | [rs28929474] |
| .0012 | PI M(MALTON) | PI, PHE52DEL ON M2 | - |
| .0013 | PI S | PI, GLU264VAL ON M1V | [rs17580] |
| .0014 | PI M(HEERLEN) | PI, PRO369LEU ON M1A | [rs199422209] |
| .0015 | PI M(MINERAL SPRINGS) | PI, GLY67GLU ON M1A | [rs28931568] |
| .0016 | PI M(PROCIDA) | PI, LEU41PRO ON M1V | [rs28931569] |
| .0017 | PI M(NICHINAN) | PI, PHE52DEL AND GLY148ARG | - |
| .0018 | PI I | PI, ARG39CYS ON M1V | [rs28931570] |
| .0019 | PI P(LOWELL) PI NULL(CARDIFF) PI Q0(CARDIFF) | PI, ASP256VAL ON M1V | [rs121912714] |
| .0020 | PI NULL(GRANITE FALLS) PI Q0(GRANITE FALLS) | PI, TYR160TER ON M1A | - |
| .0021 | PI NULL(BELLINGHAM) PI Q0(BELLINGHAM) | PI, LYS217TER ON M1V | [rs199422211] |
| .0022 | PI NULL(MATTAWA) | PI, LEU353PHE ON M1V | [rs28929473] |
| .0023 | PI NULL(PROCIDA) PI NULL(ISOLA DI PROCIDA) PI Q0(PROCIDA) | PI, 17-KB DEL | - |
| .0024 | PI NULL(HONG KONG 1) PI Q0(HONG KONG 1) | PI, 2-BP DEL, FS334TER | - |
| .0025 | PI NULL(BOLTON) PI Q0(BOLTON) | PI, 1-BP DEL | - |
| .0026 | PI PITTSBURGH 'ANTITHROMBIN' PITTSBURGH | PI, MET358ARG | [rs121912713] |
| .0027 | PI V(MUNICH) | PI, ASP2ALA ON M1V | [rs199422212] |
| .0028 | PI Z(AUGSBURG) PI Z(TUN) | PI, GLU342LYS ON M2 | - |
| .0029 | PI W(BETHESDA) | PI, ALA336THR ON M1A | [rs1802959] |
| .0030 | PI NULL(DEVON) PI Q0(DEVON) PI NULL(NEWPORT) PI Q0(NEWPORT) | PI, GLY115SER | [rs11558261] |
| .0031 | PI NULL(LUDWIGSHAFEN) PI Q0(LUDWIGSHAFEN) | PI, ILE92ASN | [rs28931572] |
| .0032 | PI Z(WREXHAM) | PI, SER-19LEU | [rs140814100] |
| .0034 | PI NULL(HONG KONG 2) PI Q0(HONG KONG 2) | PI | - |
| .0035 | PI NULL(RIEDENBURG) | PI, DEL | - |
| .0036 | PI KALSHEKER-POLLER | PI, G-A, 3-PRIME UTR ENHANCER | - |
| .0037 | PI P(DUARTE) | PI, ASP256VAL | - |
| .0038 | PI NULL(WEST) PI Q0(WEST) | PI, IVS2DS, G-T, +1 | - |
| .0039 | PI S(IIYAMA) | PI, SER53PHE | [rs55819880] |
| .0040 | PI Z(BRISTOL) | PI, THR85MET ON M1V | [rs199422213] |

Fraizer et al., 1989 [27] studied the molecular defect in M(Malton), a deficiency allele which, like the Z allele, is associated with hepatocyte inclusions and impairs secretion. They found that the M(Malton) allele contains a deletion of the codon for 1 of the 2 adjacent phenylalanine residues (amino acid 51 or 52 of the mature protein). Judging from the haplotype data, the M(Malton) mutation must have derived from the normal M2 allele. Deletion of the 1 amino acid would be expected to shorten 1 strand of the beta-sheet, B6, apparently preventing normal processing and secretion. Curiel et al., 1989 [28] also showed that the M(Malton) allele differs from the normal M2 allele by deletion of the entire codon (TTC) for residue phe52. They demonstrated abnormal intracellular accumulation of newly synthesized AAT protein in a homozygote that also showed, on liver biopsy, inflammation, mild fibrosis, and intrahepatocyte accumulation of the protein. PI S (.0013 PI, GLU264VAL ON M1V [dbSNP:rs17580]) was described by Owen and Carrell 1976 [29] and Yoshida et al., 1977 [30] who found substitution of valine for glutamic acid at position 264 in the S variant of alpha-1-antitrypsin (Long et al. 1984 [31]). Curiel et al., 1989 [32] concluded that the S-type AAT protein is degraded intracellularly before secretion. PI*S homozygotes are at no risk of emphysema, but compound heterozygotes with Z or a null allele have a mildly increased risk. Because of the high frequency of the PI*S allele (0.02-0.04 in US Caucasians), such compound heterozygotes are relatively frequent.

We described first allelic variants of SERPINA1 gene in Macedonians with reverse hybridization method and concentration of AAT in serum of eleven patients with different combinations of alleles. The number of investigated patients is very small and we need more genotyping of SERPINA1 gene in order to analyze frequency and/or association studies.

In conclusion, a diagnosis in the case of a suspicion of AAT deficiency is carried out by measuring the alpha-1-antitrypsin level in blood and by genotyping of alpha-1-antytripsin allele. The importance of early diagnosis or diagnosis early in life, resides in the possibility of smoking cessation and treatment of pulmonary disease which could significantly decrease the morbidity associated with this chronic disease.

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