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Alpha$_1$-antitrypsin alleles in patients with pulmonary emphysema, detected by DNA amplification (PCR) and oligonucleotide probes

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ABSTRACT: Alpha$_1$-antitrypsin (AAT) deficiency is a serious predisposing factor for the development of pulmonary emphysema.

Twelve representative Danish families were studied. AAT typing was performed as a comparative study between the traditional protein typing by isoelectrical focusing and the deoxyribonucleic acid (DNA) technique of enzymatic amplification and subsequent typing with radioactively labelled oligonucleotide probes.

On the basis of clinical and radiological signs of pulmonary emphysema, 25 patients were selected. AAT typing was performed by use of the two techniques in combination, in search for new point-mutations among the patients. Results obtained with the two techniques were discordant in one patient, suggesting an unknown variant.

The unexpectedly high PiZ frequency of 0.22 found in the study group is discussed.

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A principal theory explaining the development of pulmonary emphysema gives a protease-antiprotease imbalance a major role [1]. Elastase is a serine protease, formed in the azurophilic granules of the mature neutrophils, with the ability to cleave native elastin [2, 3]. Inflammation in the lung results in the release of elastase, which in the tissue is inactivated by complex formation with the protease inhibitors [4]. The most potent inhibitor in humans is the serine protease inhibitor alpha$_1$-antitrypsin (AAT or Pi), which is almost exclusively secreted by liver cells (for review see [5]). The inactivation of the elastase is normally so efficient that no activity is left in the pulmonary tissue [6, 7]. However, deficiency of AAT may leave some of the elastase uninhibited, resulting in destruction of the elastic tissue in the alveolar wall and subsequent development of emphysema of the lungs. Many reasons are given for development of pulmonary emphysema, and one important factor is the genetic deficiency of AAT. However, some individuals with AAT deficiency do not develop pulmonary function impairment. Recent investigations have shown that other factors contribute to the serious clinical course. These are cigarette smoking, asthma, lower respiratory tract infections and possibly some familial factors [8, 9]. LAURELL and ERIKSSON [10] first described the relationship between deficiency of AAT and heredity, by AAT typing of families with pulmonary emphysema. Later investigations have shown, that both alleles are expressed codominantly, and that the locus is situated on chromosome 14 [11].

The AAT locus (Pi locus) is very polymorphic with more than 60 protein variants described [12]. The most frequent variants causing AAT deficiency are the Z and S variants. The allele frequencies of PiZ and PiS are 0.02 in Northern Europe [13, 14]. The protein coded for by the Z allele aggregates within the liver cells, resulting in a serum AAT concentration equivalent to about 15% of that associated with the normal M allele [6]. The product of the S allele gives a serum concentration of 60% of that associated with the normal allele. Individuals with the phenotype PiZ are at great risk of developing pulmonary emphysema as about 85% have pulmonary features of this disease [5]. PiS is only a risk factor in combination with another deficiency causing allele [6]. Upon isolation and sequencing of the AAT gene, it has been found that the Z and S alleles are formed by two different point-mutations [15, 16]. The Z allele appears to be a guanine to adenine mutation in the fifth exon (coding region), resulting in a change in the amino acid sequence from glutamic acid to lysine (fig. 1). The S allele appears to be an adenine to thymine mutation in the third exon (coding region), resulting in a change in the amino acid sequence from glutamic acid to valine.

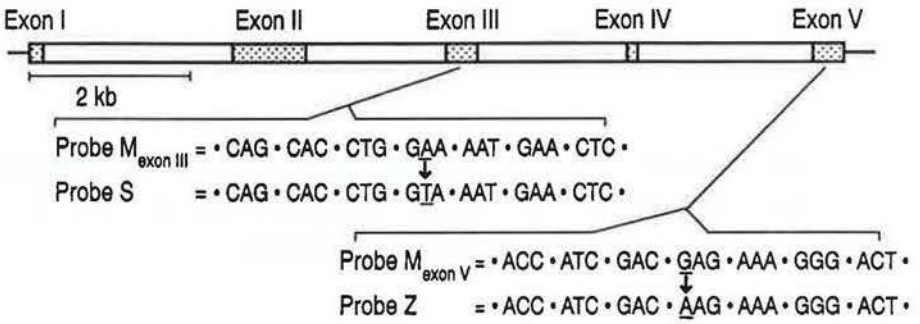

Fig. 1. – Organization of the AAT gene. The gene coding for AAT has a length of 10 kb (kilobases) and consists of five exons (coding regions) and four introns (noncoding regions). The Z and S variants are the result of changes (point-mutations) in nucleotides of the AAT gene, a G to A mutation in exon V and an A to T mutation in exon III, respectively. The four oligonucleotide probes corresponding to the nucleotide sequence of the normal gene and the gene with the Z and S point-mutations are shown. Two probes are needed for detection of each mutation, one with the sequence of the normal gene and the other with the sequence of the mutated gene. AAT: alpha$_1$-antitrypsin.

A method allowing the detection of point-mutations in the globin gene by oligonucleotide probes was published by WALLACE *et al.* [17]. The method has been applied for the identification of the Z allele in the AAT gene also [18], but is very laborious and time-consuming, and the intensities of the signals obtained are often at the limit of detection. To overcome these limitations we adopted the technique of polymerase chain reaction (PCR) to the procedure, which made it possible to detect the Z mutation by dot-blot analysis [19].

We have compared two methods of AAT typing, protein typing by isoelectric focusing and deoxyribonucleic acid (DNA) typing by PCR amplification and oligonucleotide probing, on 12 representative Danish families and 25 patients with a clinical history of pulmonary emphysema. The reliability of the methods were investigated by segregation analysis. The two techniques in combination were used for detection of new point-mutations in a group of patients with serious pulmonary emphysema.

Material and methods

Twelve Danish families with a total of 71 family members, representing all possible combinations of the three variants M, Z and S, were investigated to test the reliability of the methods.

Twenty five consecutive adult patients (12 males and 13 females), who were under 60 yrs of age and had a clinical and radiological diagnosis of emphysema of the lungs, agreed to participate in the investigation. All patients were smokers or had given up smoking within the last year. Emphysema was suspected if the patient complained of slowly progressive dyspnoea during physical performances, and if clinical examination showed an over-expanded chest with quiet breath sounds. Further diagnostic support was obtained by the following X-ray findings: chest X-rays should give evidence of hyperinflation with increased retrosternal translucency, flat, depressed diaphragms, attenuation and narrowing of peripheral vessels and, in addition, often bullous areas.

Lung function (residual volume (RV), vital capacity (VC), forced expiratory volume in one second (FEV_1)) was measured with a bell spirometer (Godart, Bilthoven, The Netherlands). All measurements were performed with the patient in the sitting position, wearing a noseclip. Normal values were adapted from QUANJER [20]. Measurements of transfer coefficient for carbon monoxide (D/VA) ($mmol \cdot s^{-1} \cdot kPa^{-1} \cdot l^{-1}$) were attempted with all 25 patients by a single breath technique with transferscreen II (Jaeger, Wurtzburg, FRG).

AAT typing

Ten millilitres of blood stabilized with edetic acid (EDTA) and 1–2 ml serum were collected from each of the 25 patients and family members. The blood and serum were stored at -20°C until used. Preparation of DNA from the frozen blood was carried out according to the procedure described by GUSTAFSON *et al.* [21]. AAT phenotyping was performed by isoelectric focusing [22].

Amplification by polymerase chain reaction (PCR)

Specific DNA segments in the AAT gene were amplified by PCR [23]. The principle of PCR is shown in figure 2. Primers were synthesized flanking each of the two mutations Z and S. One pair of primers was used for amplification of the segment of 139 base-pairs containing the site of Z mutation [24]:

1: 5'CCTGGGATCAGCCTTACAACGTGTCTCTG
2: 5'CGGGGGGGATAGACATGGGTATGGCCTCT

Another pair of primers was used for amplification of a segment of 148 base-pairs containing the site of S mutation:

1: 5'CAATGCCACCGCCATCTTCTTCCTGCCTG
2: 5'TGTGGGCAGCTTCTTGGTCACCCTCAGGT

The four oligonucleotides were mixed to allow the simultaneous amplification of both the Z and S regions.

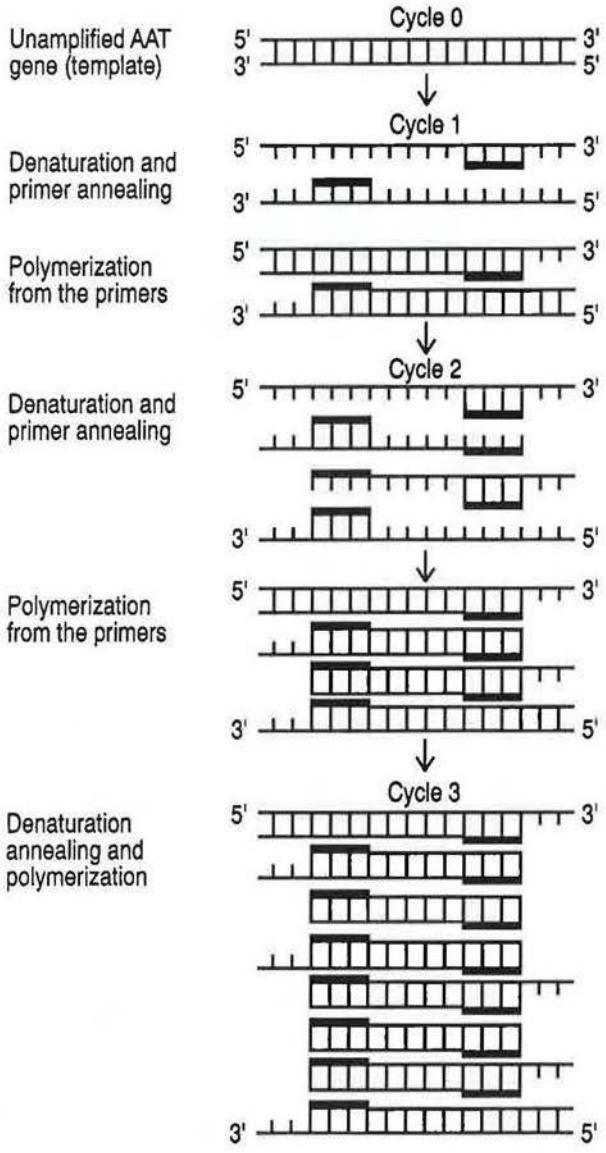

Fig. 2. – The principle of the polymerase chain reaction (PCR). The genomic deoxyribonucleic acid (DNA) provides the template strands for the synthesis of DNA from primers and mononucleotides with the enzyme Taq polymerase. Repeated cycles of high temperature template denaturation, oligonucleotide primer annealing, and polymerase mediated extension leads to amplification of DNA strands in between the primers. Two primers (29-meres), are synthesized complementary to each of the two strands of the template and flanking the region to be amplified. At a temperature of 95°C the two strands of the target DNA dissociate. At 52°C the primers anneal to the single stranded genomic DNA, and at 74°C two new DNA strands are created by extension from the 3'end of the primers. The two newly synthesized strands are complementary to the target DNA and will serve as new templates in the PCR. Repeated cycles of denaturation, primer annealing and extension will result in an exponential accumulation of the 139-bases region defined by the primers.

The reaction was performed according to the procedure recommended by Perkin Elmer Cetus (Perkin Elmer Cetus Manual), except for the concentration of the primers which were 60 pmol each, and the amount of Taq polymerase which was 1 unit for 30 cycles of amplification.

Oligonucleotide hybridization

Four 19-mer oligonucleotide probes were synthesized for identification of the two point-mutations Z and S (fig. 1) - two probes for each mutation. One probe with the sequence of the normal allele and the other probe with the sequence of the mutated allele [25]. The probes were labelled with gamma ^{32}P-adenosine triphosphate (ATP) catalysed by T4-polynucleotide kinase.

By hybridization of a probe to single-stranded genomic DNA a double-stranded DNA (duplex) molecule is formed. DNA duplexes containing nucleotide mismatches are unstable and will denature at a lower temperature than duplexes with no mismatches. At a specific temperature only duplexes with perfectly matched probes are stable [17].

The amplification product was spotted onto Zeta membranes. Four identical membranes with DNA dots were made - one for hybridization with each of the four probes. The dots were hybridized with the radioactive probes for one hour at 52°C. The membranes were washed separately in plastic bags at room temperature for 30 min to wash off the unspecifically bound probes. Stringent wash was performed for about 30 min as follows: membranes hybridized with $M_{exonIII}$, S and Z probes were washed at 62°C, and membranes hybridized with M_{exonV} probe were washed at 63°C. The probes were visualized by autoradio-graphy with Kodak XAR-5 XC-ray film for 1–2 h. A detailed description of this method is given by SCHWARTZ *et al.* [26].

Results

Segregation analysis

The Mendelian segregation in 12 representative families was tested with the DNA technique using oligonucleotide probes and compared to isoelectric focusing. The pedigree of one of these families is shown in figure 3 and the autoradiogram from DNA typing of the same family is shown in figure 4. For all of the families investigated the two types of analysis agreed and identical segregation was found.

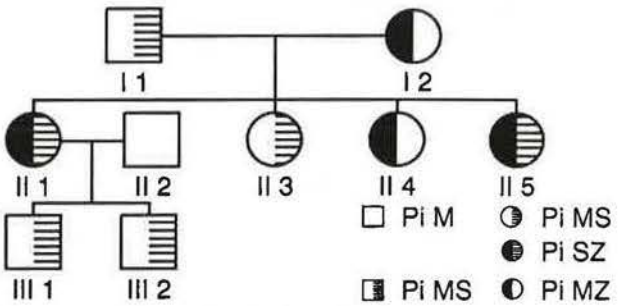

Fig. 3. – Pedigree of a family with AAT deficiency. Typing of the AAT gene at the DNA level was performed by oligonucleotide probing and at the protein level by isoelectric focusing. The index case has the AAT type PiSZ, with an S allele from her father and a Z allele from her mother. Only the S allele has passed on to her children, who both have the AAT type PiMS. AAT: alpha$_1$-antitrypsin; DNA: deoxyribonucleic acid.

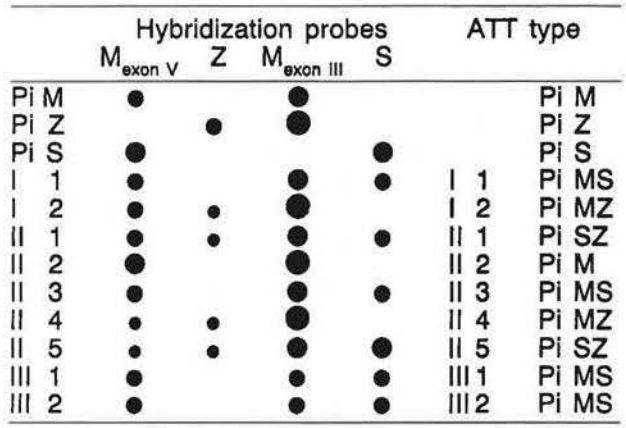

Fig. 4. – Dot-blot analysis. Three DNA controls (PiM, PiZ and PiS) are included in the analysis. The family members are identified by their number. Four identical dot-blots were prepared from the amplified DNA. Each of these was hybridized with one of the four radioactively labelled oligonucleotide probes. At 52°C both the matching and the nonmatching probes are fixed to the amplified DNA (not shown). At 62°C concerning the $M_{exonIII}$, Z and S probes, or at 63°C concerning the M_{exonV} probe nonmatching probes are removed. The genotypes revealed by the dot-blots are listed on the figure. AAT: alpha$_1$-antitrypsin; DNA: deoxyribonucleic acid.

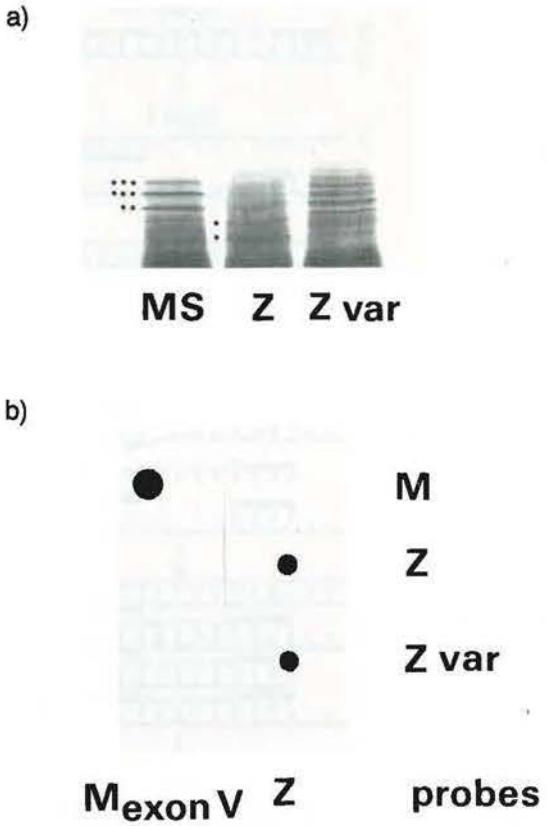

Fig. 5. – a) shows the gel of isoelectric focusing from two controls with the phenotype PiMS and PiZ and the patient PiZ var. The location of the different bands in the gel is marked by dots: · the two Z bands; ·· the S band; ··· the two M bands. b) shows a film of dot-blots visualized by autoradiography. Amplified DNA from two controls (PiM and PiZ) and the patient Z var are spotted onto two membranes. Each of the two identical membranes are hybridized with one of the two probes M_{exonV} and Z probe. DNA: deoxyribonucleic acid.

AAT typing of patients with pulmonary emphysema

The comparative study of AAT typing with isoelectric focusing and oligonucleotide probing of 25 patients with pulmonary emphysema revealed that 16 had the phenotype PiM, two were PiMS, two were PiMZ and four were PiZ. For one patient the two methods of typing gave partially different results as shown in figure 5a and b. Protein typing showed the two Z bands together with four other bands lying in between the M, S and Z bands. DNA typing with oligonucleotide probes showed a PiZ type only. The concentration of AAT in serum was 0.4 g·l^{-1}.

Table 1. – Demographic data for the 25 patients selected for pulmonary emphysema and classified according to the AAT genotype

AAT genotype	n	AAT g·l^{-1}	Age yrs	Height cm	Weight kg
MM	16	4.7 (3.0–5.8)*	52 (37–70)	169 (159–185)	53.5 (35.5–91.0)
MS	2	2.9 (2.7–3.1)	53 (41–65)	184 (176–191)	81.0 (80.0–82.0)
MZ	2	2.2 (2.1–2.2)	50 (44–56)	170 (168–172)	53.5 (49.0–58.0)
ZZ	4	0.5 (0.4–0.6)	51 (43–54)	167 (164–168)	62.0 (44.0–64.0)
PiZ?	1	0.4	43	173	75.0

The serum concentration of AAT is given as median and range in parenthesis. Control range 3–6 g·l^{-1}. AAT: alpha$_1$-antitrypsin. *: n=15.

Table 2. – Lung function indices in 25 patients with pulmonary emphysema classified according to the AAT genotype

AAT genotype	n	Lung function % predicted TLC	RV	FEV_1	D/VA
MM	16	104 (74–155)	187 (83–333)	28 (10–84)	47 (17–85)*
MS	2	101 (75–127)	168 (116–219)	31 (21–42)	-
MZ	2	97 (91–103)	125 (81–169)	66 (43–90)	64 (51–77)
ZZ	4	96 (82–126)	164 (102–261)	31 (17–34)	53 (44–59)**
PiZ?	1	96	222	14	-

All values are listed in percentage of predicted normal values, median and range in parenthesis. AAT: alpha$_1$-antitrypsin; TLC: total lung capacity; RV: residual volume; FEV_1: forced expiratory volume in one second; D/VA: transfer coefficient for carbon monoxide. *: n=10; **: n=3.

In tables 1 and 2 the results of lung function are shown. The measurements revealed, for all patients, a severe obstructive pulmonary disease. Mean FEV_1 was 29% (range 10.4–89.8%) of predicted normal value, and RV was relatively increased. Diffuse fibrotic changes were observed on the chest X-rays of the PiZ patients in agreement with the low total lung capacity (TLC) of these patients. One possible explanation for this observation could be the presence of chronic inflammation. A satisfactory co-operation and performance of the single breath CO transfer test was only possible in 15 patients because of severe impaired lung function.

Discussion

For a long time, isoelectric focusing has, together with determination of the AAT concentration in serum, been the method of choice for AAT phenotyping. The technique is rather simple but interpretation of the bands can be difficult and demands skilled personnel. The method can identify about 60 protein variants including the deficient AAT types, PiZ and PiS, which compose the vast majority of the disease associated variants. Typing with oligonucleotide probes after PCR is technically more demanding than protein typing, but the interpretation is simple. It is a method for detection of specific point-mutations and the specificity is high. However, mistyping is possible when nonexpected point-mutations are present. A point-mutation at the site of hybridization with the probes will give rise to unstable binding of both probes, and a point-mutation at the hybridization site of one of the primers might make the amplification of this allele inefficient. In both cases only the allele without the nonexpected point-mutation will be typed.

Nevertheless, it is a very rapid and safe method for detection of specific point-mutations. The generation of the signal lasts about 1 h and the analysis can be totally finished within 24 h, compared to 2–3 days by isoelectric focusing. As any cell can be used, it is ideal for prenatal diagnosis [26].

Among other methods available for specific detection of point-mutations in the AAT genome, ABE *et al.* [27] have adopted DNA amplification by PCR together with ribonuclease (RNase) A cleavage methodology [27]. The advantage of this method compared to the use of oligonucleotide probes is the ability to analyse a fragment of 0.33 kb composing exon V and flanking sequences. Although experience has shown that the ability of the RNase to cleave mismatches depends specifically on the mutation, the technique should be useful for screening the AAT gene for new mutations.

In our experience, the isoelectric focusing should be used as a screening method and the DNA technique as a verification of the deficient variants. If the result of the two methods disagree, sequence analysis should be performed.

Besides the Z and S alleles, very rare deficient AAT types have been described, and within the last few years the genetic cause of some of these alleles has been determined. Two groups of variants have been characterized, *i.e.* the null variants and the low level deficiency variants. For the null variants (Pi QO) [28–31] sequence analyses have revealed changes in the nucleotide sequence of the genes, resulting in premature termination of transcription (stop codons). Consequently, no active protein is produced from the liver cells.

In the low level deficiency variants [32–34] a very low AAT concentration in serum is found, and by isoelectric focusing the bands have migrated to the position of the normal M bands. Sequence analysis has shown that two of these deficiency alleles (M Procida and M Herleen) have normal nucleotide sequence except for a single point-mutation [32, 34], whereas the M Malton allele has a triplet deletion. In all of these variants changes are found in the coding region of the AAT gene, and subsequent changes in the tertiary structure of the protein are the probable mechanism for the deficiency.

In our investigation of 25 patients the two AAT typing methods showed a discrepancy in one case. Isoelectric focusing revealed a Z allele and an unknown allele, whereas DNA typing indicated only a Z allele. As the point-mutations of the described low deficient variants are all situated outside the amplified and tested DNA sequence and typing with oligonucleotide probes indicated no M alleles, we do not believe that the patient is heterozygous for one of the above mentioned very rare alleles. But the explanation could be a mistyping with oligonucleotide probes and so a new variant. In the study group of 25 patients with primary emphysema, the frequency of the Z allele was at least 0.22 compared to frequencies of 0.02 in a normal Danish population [13]. The frequency is high compared to Z allele frequencies of 0.056–0.103 in similar investigations [34–37]. The explanation of this very high frequency could be a bias in selecting the patients, which is of outstanding importance especially in small sample size. As the patients were selected because of radiological and clinical signs of emphysema and without any knowledge of the AAT level we can exclude a selection bias. However, we cannot exclude a bias because of a small sample size. Other explanations of the high PiZ frequency among the 25 patients could be the ages of the patients. LIEBERMAN *et al.* [36] indicated that patients with primary emphysema below the age of 50 yrs have a higher prevalence of AAT deficiency than older patients. By comparing the mean age of our patient with the mean age of patients in other investigations [35, 37] we do not believe this to be the explanation, as the mean ages are of the same magnitude. Technical differences could also explain the higher frequency of the Z allele. JANUS [37] used serum tryptic inhibitory capacity (STIC) and radial immunodiffusion combined with acid starch gel electrophoresis and found a frequency of PiZ of 0.103. However, most investigators [35, 36, 38], including ourselves, used isoelectric focusing, but found frequencies of $\leq$0.056. Therefore, we do not

believe that technical differences play any role for the observed high frequency among our patients. Our conclusion is that the PiZ frequency of 0.22 in our study group can be explained by the small sample size or by stringent primary selection of the patients from clinical and radiological criteria. It is also worth mentioning, that all of the patients were smokers or had given up smoking within the last year as smoking is known to be a high risk factor of pulmonary emphysema in persons deficient of AAT.

By reviewing the result of 12 studies concerning AAT typing of patients with chronic obstructive pulmonary diseases (COPD), BARTMAN *et al.* [35] found that 10 studies pointed to a higher risk for individuals with the phenotype PiMZ compared to PiM. Together with our study 11 of 13 studies give a higher frequency of MZ among patients with COPD compared to controls. If we assume that the probability of observing a higher MZ frequency is equal to observing a lower MZ frequency the difference is statistically significant p=0.04 (sign test, two-sided). We therefore believe that the phenotype PiMZ should be regarded as another predisposing factor for development of pulmonary emphysema.

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