

Alpha-1-Antitrypsin Deficiency in Serbian Adults with Lung Diseases

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Aim: Alpha-1-antitrypsin (A1AT) is the main inhibitor of neutrophil elastase, and severe alpha-1-antitrypsin deficiency (A1ATD) is a genetic risk factor for early-onset emphysema. Despite the relatively high prevalence of A1ATD, this condition is frequently underdiagnosed. Our aim was to determine the distribution of the A1ATD phenotypes/alleles in patients with lung diseases as well as in the Serbian population. **Methods:** The study included the adults with chronic obstructive pulmonary disease (COPD) ($n=348$), asthma ($n=71$), and bronchiectasis ($n=35$); the control was 1435 healthy blood donors. The A1ATD variants were identified by isoelectric focusing or polymerase chain reaction-mediated site-directed mutagenesis. **Results:** PiMZ heterozygotes, PiZZ homozygotes, and Z allele carriers are associated with significantly higher risk of developing COPD than healthy individuals (odds ratios 3.43, 42.42, and 5.49 respectively). The calculated prevalence of PiZZ, PiMZ, and PiSZ was higher in patients with COPD (1:202, 1:8, and 1:1243) than in the Serbian population (1:5519, 1:38, and 1:5519). **Conclusion:** The high prevalence of A1ATD phenotypes/allele in our population has confirmed the necessity of screening for A1ATD in patients with COPD. On the other hand, on the basis of the estimated number of those with A1ATD among the COPD patients, it is possible to assess the diagnostic efficiency of A1ATD in the Serbian population.

Introduction

ALPHA-1-ANTITRYPSIN (A1AT) is an acute-phase glycoprotein that is mainly synthesized in hepatocytes and subsequently secreted into the plasma. Besides the liver, small quantities of A1AT are produced by alveolar macrophages, circulating monocytes, and intestinal-, renal-, and lung-derived epithelial cells (Cichy *et al.*, 1997). Extrahepatic synthesis of A1AT is important in preventing tissue damage in the site of inflammation or injury. The target proteases of A1AT originate from azurophilic granules of polymorphonuclear neutrophils. The main physiological role of A1AT is protection of the lower respiratory tract by inhibition of neutrophil elastase (NE), which is released from triggered neutrophils. Moreover, A1AT has anti-inflammatory properties (Janciauskiene *et al.*, 2007; Pott *et al.*, 2009; Bergin *et al.*, 2010), and inhibits the lung endothelial cell apoptosis, which is crucial for the maintaining pulmonary homeostasis (Petrache *et al.*, 2006).

A1AT is encoded by the *SERPINA1* gene (serpin peptidase inhibitor, clade A), located in the proteinase inhibitor (Pi)

locus on the long arm of chromosome 14q32.1, and shows a codominant pattern of inheritance. *SERPINA1* is highly polymorphic, and more than 125 single-nucleotide polymorphisms (SNPs) have been reported in public SNP databases (Entrez SNP). Protein variants of A1AT are classified by the Pi (Protease inhibitor) system. Most common PiM variants have a normal serum level and functional activity to inhibit NE.

The main characteristic of hereditary alpha-1-antitrypsin deficiency (A1ATD) is a low level of circulating A1AT. The post-translational modification of the A1ATD variants is altered. Misfolded protein has a high tendency to form oligomers and larger polymers that are retained in the hepatocytes, and consequently leads to the reduction of circulating A1AT (Carrell and Lomas, 1997). Because the integrity of lung alveoli depends on the proper levels of circulating A1AT, severe A1ATD was identified as a genetic risk factor for early-onset emphysema. Thus, the imbalance of protease-antiprotease leads to A1ATD-related emphysema due to the excessive activity of NE, which degrades the insoluble elastin and the other components of the extracellular matrix in the lower respiratory tract.

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Several mutations associated with A1ATD have been identified, and the most common are the Z and S alleles. The plasma levels of A1AT in PiZZ homozygotes are severely reduced (10–15% of normal), whereas in the PiSS, PiMS, PiMZ, and PiSZ heterozygotes, the reduction is mild (35–70%).

The aim of this study was to estimate the prevalence of A1ATD in the patients with lung disease. To our knowledge, these data for our population do not exist. Two A1ATD epidemiological studies (De Serres, 2002; Blanco *et al.*, 2006) have included data for the Serbian population. Our intention was to update the data and estimated number of A1ATD individuals in the Serbian population.

Methods

Study subjects

The study included adult patients with lung disease admitted to the Clinic for Pulmonary Diseases Clinical Centre of Serbia, Belgrade, Serbia, and the Zvezdara University Medical Center. The study included 348 patients with chronic obstructive pulmonary disease (COPD), 71 with asthma, and 35 with bronchiectasis. The control group included 1435 healthy blood donors. The diagnosis of COPD was established based on medical history, physical examination, pulmonary function tests, blood gas analyses, and chest radiography, according to the Global Initiative for Chronic Obstructive Lung Disease. Patients with bronchiectasis had symptoms, including regular sputum production, chronic cough, and a history of recurrent lower respiratory tract infections. The diagnosis of bronchiectasis was established by chest high-resolution computed tomography. Pulmonary function tests (forced expiratory volume in 1 s [FEV₁] and forced vital capacity) were performed. The diagnosis of asthma was based on the history of attacks of wheezing associated with shortness of breath and spirometric evidence of a bronchodilator response. The protocol has been approved by the local research ethics committees, and informed consent was obtained from all participants of the study.

Methods

Blood samples were taken from all patients. Sera were separated by centrifugation and stored at –80°C until analyzed. For detection of A1ATD variants, isoelectric focusing or

polymerase chain reaction (PCR)-mediated site-directed mutagenesis (PSM) methods were employed, depending on the method available in the laboratory at a particular time.

Isoelectric focusing method (pH range 4.2–4.9; Pharmacia LKB Biotechnology) (Kishimoto *et al.*, 1990) was performed with an LKB 2117 Multiphor system. Serum samples were pretreated by dithioerythritol, and 0.2-mm-thin polyacrylamide gels were casted by using the Pharmacia LKB Ultramould Gel Casting Unit (Pharmacia).

Genomic DNA was extracted from peripheral blood using a GFX™ Genomic Blood DNA Purification Kit (Amersham Biosciences). The presence of the most common mutated A1AT alleles, Z and S, was detected by the PSM method, as it was previously described (Lucotte and Sesboüé, 1999). In summary, fragments containing Z and S mutations were coamplified; PCR products were digested with *TaqI* (New England BioLabs), separated on a 4% agarose gel, and visualized by ethidium bromide staining. Genotypes were scored without knowledge of the sample phenotypes by two independent observers.

Statistical analysis

The differences in the frequencies of A1AT phenotypes and alleles between patients and controls were analyzed using the χ^2 test (2 × 2 contingency table). The Fisher exact test was used when $n < 5$. *p*-Values of <0.05 were considered as significant. For statistical analysis, Statistica 7.0® software was used.

Results

Frequencies of A1ATD phenotypes/alleles in patients with lung disease and controls are presented in Table 1. Significantly higher frequencies of PiMZ, PiZZ, and Z alleles were obtained only in COPD patients. Our data showed that PiMZ heterozygotes have 3.4-times higher risk of developing COPD, while a severe A1ATD in PiZZ homozygotes is associated with very high risk for COPD, 42-times higher than individuals without this phenotype. The carriers of the Z allele have 5.5-times higher risk of developing COPD than the carriers of other alleles.

Estimated prevalence and the number of A1ATD patients with COPD, and A1ATD individuals in the Serbian population without COPD are given in Table 2. The estimation was made based on the prevalence of COPD in the Serbian

TABLE 1. ALPHA-1-ANTITRYPSIN DEFICIENCY PHENOTYPES AND ALLELES IN PATIENTS WITH LUNG DISEASES AND IN CONTROL GROUP (FREQUENCIES/NUMBER)

	COPD (n = 348)	Asthma (n = 71)	Bronchiectasis (n = 35)	Control (n = 1435)	Odds ratio (95% CI)
Phenotypes					
MM	0.8764/305 ^a	0.9577/68	0.9715/34	0.9603/1378	0.31 (0.20–0.47)
MZ	0.0833/29 ^a	0.0281/2	0.0286/1	0.0258/37	3.43 (2.08–5.67)
ZZ	0.0287/10 ^a	0	0	0.0007/1	42.42 (4.41–332.55)
MS	0.0115/4	0.0140/1	0	0.0132/19	—
Alleles					
M	0.9239/643 ^a	0.9789/139	0.9857/69	0.9798/2812	0.25 (0.17–0.37)
Z	0.0704/49 ^a	0.0141/2	0.0143/1	0.0136/39	5.49 (3.58–8.44)
S	0.0057/4	0.0070/1	0	0.0066/19	—

^aSignificant difference compared to control ($p=0$).
COPD, chronic obstructive pulmonary disease; CI, confidence interval.

TABLE 2. ESTIMATED PREVALENCE AND NUMBER OF ALPHA-1-ANTITRYPSIN DEFICIENCY IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND IN SERBIAN POPULATION WITHOUT CHRONIC OBSTRUCTIVE PULMONARY DISEASE

	Observed number	Observed frequency	Expected number	Expected frequency	Estimated prevalence ^a	Estimated number ^b
COPD (n = 348)						
Phenotypes						
MM	305	0.8764	297.05	0.8535	/	/
MZ	29	0.0833	45.27	0.1300	1:8	55,578
ZZ	10	0.0287	1.72	0.0049	1:202	2112
MS	4	0.0115	3.66	0.0105	1:95	4493
SZ	0	0	0.28	0.0008	1:1243	344
SS	0	0	0.01	0.0001	1:34,800	12
Alleles						
M	643	0.9239				
Z	49	0.0704				
S	4	0.0057				
Control (n = 1435)						
Phenotypes						
MM	1378	0.9603	1377.6	0.9600	/	/
MZ	37	0.0258	37.97	0.0264	1:38	177,108
ZZ	1	0.0007	0.26	0.0002	1:5519	1212
MS	19	0.0132	18.37	0.0128	1:78	85,685
SZ	0	0	0.26	0.0002	1:5519	1212
SS	0	0	0.06	0.0000	1:23,917	280
Alleles						
M	2812	0.9798				
Z	39	0.0136				
S	19	0.0066				

^aPrevalence of COPD in Serbia is 6%; for the calculation, the estimated number of 427,240 patients with COPD was used.

^bPopulation of Serbia consists of 7,120,666 inhabitants; for the calculation, the estimated number of 6,693,426 individuals without COPD was used.

population of 6% (Franchi, 2009), and the size of Serbian population (Official Website of the Serbia Government, www.srbija.gov.rs/pages/article.php?id=6). According to these data, the estimate is that of 7,120,666 Serbian citizens, 427,240 individuals will be affected with COPD.

The frequency of the S allele in our population was low (<1%), and the association with COPD was not observed. Nevertheless, the S allele and S allele carriers are presented in Table 2, due to the association between S allele carriers and COPD (Dahl *et al.*, 2005). In this meta-analysis, the risk of COPD is approximately tripled in PiSZ, and significantly increased in PiMS carriers. Although the risk for COPD in the PiMS subgroup adjusted for smoking or COPD based on spirometry were not increased, the authors could not completely exclude the possibility of PiMS genotype importance in the pathogenesis of COPD.

The prevalence of A1ATD phenotypes in Serbian and European patients with COPD and corresponding populations is given in Table 3. This table presents the current data for the Serbian population, as well as the estimated prevalence of the PiS and PiZ gene calculated by Blanco *et al.* (2006) based on data from 21 European countries. The data obtained in a recent study conducted in Ireland (Carroll *et al.*, 2011) are also included.

Discussion

The risk of COPD development caused by hereditary A1ATD is inversely correlated with level of serum A1AT. The American Thoracic Society/European Respiratory Society

Statement (2003) recommended quantitative testing of A1AT in all patients with COPD, in those with asthma characterized by incompletely reversible airflow obstruction and in patients with bronchiectasis without evident etiology. Severe A1ATD phenotypes, PiZZ, PiSZ, and PiNull, or carriers of some other rare variants are significantly associated with panlobular emphysema. Typically, clinical manifestations of A1ATD-related emphysema are dilatation or destruction of all lower lobules. The PiMZ heterozygotes have mild reduction of the circulating A1AT. Although, the data regarding the risk of COPD in PiMZ individuals are inconsistent, a significant number of representative studies have shown the association between PiMZ and COPD. These studies have shown the

TABLE 3. PREVALENCE OF ALPHA-1-ANTITRYPSIN DEFICIENCY PHENOTYPES IN SERBIA AND IN EUROPE

Phenotypes	Populations				
	COPD Serbia-this study	Serbia-this study	Serbia ^a	Europe ^a	Ireland ^b
MZ	1:8	1:38	1:40	1:36	1:25
ZZ	1:202	1:5519	1:6165	1:4727	1:2104
MS	1:95	1:78	1:77	1:16	1:10
SZ	1:1243	1:5519	1:5945	1:1051	1:424
SS	1:34,800	1:23,917	1:22,931	1:934	1:341

^aBlanco *et al.* (2006).

^bCarroll *et al.* (2011).

decrease in elasticity and deterioration of lung function parameters (Tarján *et al.*, 1994), greater rate of decrease in FEV₁ (Dahl *et al.*, 2002), higher susceptibility to the development of airflow obstruction (Sørheim *et al.*, 2010), and increased odds of COPD in PiMZ individuals (Hersh *et al.*, 2004). On the other hand, the mild deficiency in the PiMZ heterozygote is sufficient for antielastase defense of the lung and contributes to a lower-penetrance PiMZ phenotype than PiZZ.

Current results in Serbian adults confirmed that the severe A1ATD (PiZZ), mild A1ATD (PiMZ), and Z alleles represent high risk factors for COPD (odds ratios [ORs]: 42.42, 3.43, and 5.49, respectively). Our data correspond to the data from the study by Koyama and Geddes (1998). In that study, compiled data from 10 case-control studies from different populations were presented, and ORs for PiZZ and for PiMZ were 38.85 and 2.48, respectively. Furthermore, in the current study, 2.9% of COPD patients were PiZZ, which corresponds with the report of the World Health Organization (1997) that 2–3% of all emphysema patients are PiZZ. Therefore, WHO recommended screening for A1ATD in patients with COPD and emphysema. In addition, we observed that among COPD patients, 8.3% were PiMZ, which is similar to the data obtained in Danish patients with clinically established COPD (6.3%) (Dahl *et al.*, 2001). However, screening of an unselected population with COPD, asthma, and bronchiectasis in Germany (Wencker *et al.*, 2002) detected that frequencies of PiMS and PiMZ were similar to the healthy population, and the PiZZ homozygotes in patients with asthma or COPD were very rare (<0.1%). The most common reasons that lead to the inconsistent results in COPD-A1ATD association studies are differences in population genetics, in stratification of COPD patients and in COPD phenotype definition. Regarding PiMZ heterozygosity, further reason for the discrepancies between studies is that the phenotype is often unrecognized due to the mild symptoms of COPD.

Although emphysema represents a predominant clinical manifestation of COPD in A1ATD, investigation of other airway diseases associated with the A1ATD is in progress. Published data regarding the association between bronchiectasis and A1ATD are controversial (Cuvelier *et al.*, 2000; Parr *et al.*, 2007). Cuvelier *et al.* (2000) concluded that bronchiectasis may be a consequence of emphysema in PiZZ patients rather than a primary effect. The influence of irreversible airflow obstruction in patients with severe A1ATD on the prevalence of asthma is also unclear. Recent review (Eden, 2010) was shown that the asthma signs and symptoms were common in A1ATD individuals with or without COPD, because A1ATD itself predisposes for hyper-responsiveness of the airway. We did not find association between asthma and bronchiectasis with A1ATD phenotypes or alleles. In relatively small number of patients with asthma and bronchiectasis, the deficient Z allele was about five-times less frequent than in COPD patients, and almost equal with the control. Therefore, we can assume that A1ATD is not dominant in the pathogenesis of these lung diseases.

Although epidemiological studies have revealed a large number of individuals with A1ATD worldwide (De Serres, 2002), this condition is often undiagnosed, and exact prevalence of A1ATD in most population remains unknown. Also, study about psychosocial and ethics issues faced by adults at the risk for A1ATD has revealed that the education and diagnosis of A1ATD need to be improved (Klitzman, 2009).

Globally, A1ATD affects all major racial subgroups. There are at least 116 million carriers (PiMZ and PiMS) and 3.4 million deficiency allele's combinations (PiSS, PiSZ, and PiZZ) worldwide (De Serres, 2002). Most of A1ATD cases are misdiagnosed as COPD or nonresponsive asthma. As a result, long delays between the presentation of first symptoms and the correct diagnosis are common (Stoller *et al.*, 2005). The main causes of this situation are the presence of similar respiratory symptoms to those seen in asthma or COPD, and sporadic occurrence of symptoms such as shortness of breath, cough, excess sputum production, reduced ability to exercise, and wheezing. Therefore, it is important to estimate the prevalence of A1ATD to predict the number of potential users of medical services and also to evaluate the efficacy of A1ATD diagnostics in a particular population. All this could improve the early detection of A1ATD and early prevention of symptoms. In the current study, we attempted to estimate the prevalence of A1ATD in 348 of COPD patients, as well in 1435 control subjects. Assuming the Hardy-Weinberg equilibrium in a population of Serbia, calculated prevalence of PiZZ, PiMZ, PiMS, and PiSZ were 1:5519, 1:38, 1:78, and 1:5519, respectively. Prevalence for the Z allele carriers in our population corresponds to the prevalence in the total European population and previously analyzed data of the Serbian population published in the study by Blanco *et al.* (2006) (Table 3).

The prevalence of the S allele varies greatly in different European populations. Current study confirmed lower prevalence of the S allele in our population (0.0066) in comparison to the total European population (0.0330). The prevalence of the S allele in our population is most in correlation with the neighboring Romanian population (0.0055) (De Serres *et al.*, 2007). The recent data (Carroll *et al.*, 2011) demonstrated that A1ATD in Ireland is among the highest in the world. In the population of Ireland, a nearly two-times higher frequency of homozygotes and 10-times higher frequency of heterozygotes for the PiZ allele, in comparison to our population, were reported.

We obtained significantly higher prevalence of PiZZ, PiMZ, and PiSZ in COPD patients than in non-COPD subjects. Moreover, one of the eight COPD patients in Serbia has PiMZ, and 1 of 202 has the PiZZ genotype. If we take into account the prevalence of COPD in our population, as well the expected number of A1ATD, we estimate that 55,578 PiMZ, 2112 PiZZ, 4493 PiMS, and 344 PiSZ individuals would clinically manifested COPD. These data are an approximation. It must be taken into consideration that only 0.35% of severe A1ATD (PiZZ and PiSZ) are recognized (Luisetti and Seersholm, 2004), whereas data for the mild A1ATD (PiMZ, PiMS) do not exist.

Based on the estimates of the total number of the Z homozygotes and heterozygotes in our population, we calculated that the 24% of total number of PiMZ and even 63% of PiZZ should show clinical symptoms of COPD. The case-detection program in Spain (De la Roza *et al.*, 2005) identified 0.37% PiZZ individuals in a population of COPD patients. Taking that estimated prevalence of COPD in Spain is 9%, they speculated that between 35% and 60% of all PiZZ develop COPD. Although the prevalence of COPD in our population is lower (6%) than in the Spanish (9%), prediction of PiZZ individuals that could develop COPD is comparable.

In conclusion, current study reveals that the prevalence of A1ATD in the Serbian population is comparable to data for

total European population. The other finding is a high prevalence of PiZZ and PiMZ among COPD patients, which confirms the need for early screening for A1ATD in patients with mild and severe symptoms of COPD. Current data allow the comparison of the estimated number of A1ATD patients with clinical manifestation of COPD and those who are actually diagnosed by physician, to assess the efficiency of detection of A1ATD. Since there is no national register of A1ATD patients in Serbia, our data could represent a good starting point to justify the need for such register. An early detection of COPD caused by A1ATD is of great importance, as in the modification of life style, and the application of therapeutic approaches could modulate the occurrence of severe symptoms of COPD or alleviate them.

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Author Disclosure Statement

No competing financial interests exist.

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