Romanian Pilot Study of Alpha-1 Antitrypsin Detection-Feasibility and Challenges

ANA MARIA ZAHARIE[1,2]*, SABINA ANTONIU[3], JOANNA CHOROSTOWSKA WYNIMKO[4], FLORIN DUMITRU MIHALTAN[1,2], OANA CLAUDIA DELEANU[1,2], BEATRICE MAHLER[1,2], LAVINIA DAVIDESCU[5], OANA CRISTINA ARGHIR[6], RUXANDRA ULMEANU[2,5]

[1]Carol Davila University of Medicine and Pharmacy, Medicine Faculty, 37 Dionisie Lupu Str., 030167, Bucharest, Romania

[2] Marius Nasta Institute of Pneumology, 90 Viilor Str. , 050152 Bucharest, Romania

[3]University of Medicine and Pharmacy Grigore T Popa, 2nd Department of Medicine, Nursing/Palliative Care, 16 Universitatii Str, 700337, Iasi, Romania

[4]National Institute of Tuberculosis and Lung Diseases in Warsaw, Plocka 26 str, 01-138 Warsaw, Poland

[5]University of Medicine Oradea, 10, 1 Decembrie Sq., Oradea, Romania

[6]Ovidius University of Constana, Medicine Faculty, 4th Clinical Department, 124 Mamaia Blvd, 900527 Constanta, Romania

Alpha-1 antitrypsin deficiency (AATD) is an underdiagnosed genetic disorder that manifests primarily through pulmonary and hepatic impairment. In Romania, a targeted detection program testing for AATD was implemented between October 2012 and October 2016. A cohort study enrolled all patients with indication for AATD screening (lung or liver disease, adults and children, index and non-index cases). Testing methods were mainly represented by isoelectric focusing, genotyping and/or sequencing. 620 patients (21 children) were tested (median age 50.0±16.4 years, 58.1% men), 91.9% of proved normal. A total of 50 patients were identified to be carrying a modified genotype (26 men). Hardy-Weinberg equilibrium was used for assessing the frequency of the genetic abnormalities: 1/1.08 PiMM, 1/32 PiMS, 1/28 PiMZ, 1/48 M-rare allele heterozygote, 1/3906 PiSS, 1/2770 PiZZ, 1/1000 PiSZ and 1/12346 for a Z-rare allele heterozygote. Severe AATD was present with a 1% frequency. Prevalence of abnormal genotypes estimated for each at risk category was greater in neonate hepatitis (100%), bronchitis (20%) and adult liver cirrhosis (33.3%). In conclusion, a targeted AATD detection program with this formula is feasible in Romania and will be continued with the implementation of a national registry.

Keywords: alpha-1 antitrypsin deficiency, targeted detection, screening, rare genetic disease

Alpha-1 antitrypsin deficiency (AATD) is a rare autosomal co-dominant disorder that manifests primarily through pulmonary and hepatic impairment. Lung involvement is the most common manifestation of the disease, with panacinar emphysema, as pathogenic hallmark, and chronic obstructive pulmonary disease (COPD), considered to be inherited, as a very suggestive clinical expression of AATD[1]. Alpha-1 antitrypsin (AAT), the deficient protein involved in the pathogenesis of this genetic disease, is an acute phase protein and the main inhibitor of neutrophil elastase. A low AAT serum concentration (caused by a mutated gene) is responsible for early lung disease, such as emphysema, COPD, bronchiectasis [2] and it is correlated with progressive lung disease [3], while the polymerization of pathologic AAT (as a result of some of the genetic variants in the hepatocytes) is responsible for the liver disease [1]. AAT synthesis is controlled by the SERPINA1, a gene on the 14th chromosome. More than 120 variants of the Pi (protease inhibitor) phenotype have been described [4]. The normal allele is M, while the most common abnormal alleles are Z and S. Worldwide distribution of alleles differs according to the tested allele: Z type concentrated near the Scandinavian Peninsula and S mostly identified in the Iberian Peninsula, South Africa and South America [4]. The first identification of an AATD patient was in 1963 [5].The first screening program was conducted in Sweden in 1972 [6,7]. Despite numerous efforts and screening recommendations published by the World Health Organization [8], a very low proportion of patients had been identified. Yet, AATD remains underdiagnosed and undertreated, with a mean percentage of cases identified from cases estimated at 4% [9].AATD meets the requirements for inclusion in a screening program: it has significant impact on the health status, it is easily identifiable and it is associated with increased morbidity and mortality if untreated. During 2010-2012, Romania was part of AATD European Targeting project among 11,648 patients with respiratory diseases, enrolled from 13 countries (1,866 of them from Romania). The proportion of severe AATD varied from 3.2% in Croatia to 0% in Bosnia and Herzegovina, Estonia, Macedonia and Slovenia, while Romania had a percentage of 0.3% severe AATD cases [10]. Before implementing a screening program, the AATD prevalence in Romania was estimated through various methods, such as the Hardy-Weinberg equilibrium [11–13] or the multivariate interpolation method [4], and it ranked in the low frequency zone for both Z and S alleles. AATD is, actually, the only genetic form of COPD, where genetic testing is recommended by existing guidelines [1,14].

Experimental part

This AATD targeted detection program was conducted among the risk population from Romania, in collaboration with The National Institute of Tuberculosis and Lung Diseases (NITBLD), Warsaw, Poland. This collaboration started as part of an European project- Leonardo da Vinci Lifelong Learning Program *Introducing standards of the best medical practice for the patients with inherited Alpha-1 Antitrypsin Deficiency in Central Eastern Europe* (2011-1-PL1-LEO04-19715) LLP-LdV/PAR/2011/RO/129). The

* email: ana_neb@yahoo.com

aim of this detection program was to identify AATD among eligible individuals and to increase the awareness and knowledge on AATD. This program used the recommendations of European Respiratory Society (ERS) /American Thoracic Society (ATS) statement from 2003 regarding testing indications and methods [1].Tests were carried out after pretesting counseling and consent informed by every screened patient or by a parent of screened children. It was approved by the local Institutional Review Board (IRB) of Romanian National Institute of Pneumology «Marius Nasta», Bucharest. Samples were investigated in the NITBLD, Warsaw, Poland, according to the algorithms for detecting AATD [15,16], in order to assess the prevalence of the main AAT genotypes detected in at-risk groups and to compare with European data. According to the latest AATD guidelines (recommending focusing on the clinical indication, regardless the AAT concentration [17], from October 2012 to October 2016, all patients at-risk (including COPD, unexplained chronic liver disease, necrotizing panniculitis, granulomatosis with polyangiitis, or unexplained bronchiectasis) were tested. Although spontaneous pneumothorax is not a classical indication for AATD testing, we consider it can have an implication as some studies [18] suggest and patients with spontaneous pneumothorax were included. Other eligible categories at risk were chronic bronchitis, rare cases of persistent symptoms, without obstruction and without any other risk factor identified, and siblings of AATD patients. According to a validated Polish protocol [19], dried blood spots (DBS) were used for collecting, transporting blood samples, for analysis and diagnosis, to NITBLD in Warsaw, Poland, the main laboratory in the Central Eastern Europe Alpha-1 Antitrypsin Network (Romania is affiliated to this network). All samples were tested for AAT concentration, then isoelectric focusing, genotyping or both. If discrepancies between AAT concentration and genotype or phenotype results were noticed, the AAT gene sequence analysis [20,21] was performed. Serum levels of AAT, with normal values ranged between 20 and 250 mg/dl, were determined by nephelometry, using an IMMAGE 800 analyzer (Beckman Coulter, USA). Isoelectric focusing was performed on agarose gel with Hydrasys electrophoresis system (Sebia) and Hydragel 18 A1AT Isofocusing kit (Sebia) for immunofixation, utilizing a specific antibody to AAT. DBS were used for extraction of genomic dezoxyribonucleic acid (DNA) and detecting the presence of Z or S allele, by a real time polymerase chain reaction (RT-PCR), method in the LightCycler 480 II instrument (Roche Diagnostics Ltd., Switzerland). Hydrolysing probes coupled with fluorescent dyes (VIC or FAM) allowed complementary assessment of the mutant variants (PiZ or PiS) from DBS using a commercially available kit, such as Extract-N-Amp Blood PCR Kits (Sigma-Aldrich) [23,24]. Sequence analysis of AAT exons 2-4 was determined by a 16-capillary 3130xl Genetic Analyzer (Applied Biosystems, USA) (Genomed, Poland). Forty five patients with evident discrepancies between low serum AAT levels and normal genotype were excluded from the present analysis, as their specimens were considered deteriorated. Detection efficiency was performed by calculating proportion of cases detected from all tested patients in each at-risk group. All collected data were registered into an Apache OpenOffice database © 2014 the Apache Software Foundation. Categorical variables were described using frequencies and percentages. Continuous variables, when not normally distributed, were expressed as medians (interquartile range (IQR).

Results and discussions

A number of 665 patients, mean aged 50.0±16.4 years, predominantly smokers (60.5%) and males (58.1%), were screened during the 4-year screening study. Genotyping combined with phenotyping had performed in 396 cases, isoelectric focusing in 214, and sequencing in 33 patients. Abnormal genotype was found in 50 patients (26 men). The main indication for testing these patients for AATD was COPD (n=23), followed by bronchiectasis (n=14), emphysema (n=5), bronchial asthma (n=5), spontaneous pneumothorax (n=3), chronic bronchitis (n=2), cirrhosis (n=1), immune hepatitis (n=1) There were 8 non-index cases (siblings of patients with AATD genotype) (table 1).

Identified genotypes detected were PiMM (91.9%), heterozygous PiMS (3.2%) and PiMZ (2.6%), PiZZ(n=3; 0.48%), PiSZ(n=1; 0.16%) and rare genotypes, either PiMRare (1.3%) or PiZRare genotype (0.3%) (Table 2). Frequencies of all genotypes detected, as well as the calculated prevalence of AAT genotypes assuming a Hardy-Weinberg equilibrum are shown in table 2 Severe AATD [detection of the PiZZ, PiSZ, PiZrare or Pi(null)(null) genotypes] was rarely diagnosed (1%). Twenty one children were tested for either gastroenterological problems (neonatal hepatitis, persistent hepatic cytolysis) or unexplained lung pathology. Six of them (28.5%) had at least one AATD allele and half had a severe deficit (two children with PiZZ and one with PiIZ).

No data on AATD prevalence in Romania were published, excepting an European study [10] which applied genetic testing alone to samples. The study reported a low frequency of deficient forms in Romania (1.9%) if the AAT serum values were decreased below 1.70 mg/dL (equivalent to 1.04 g/dL in serum), in contrast to our results, and the exclusivity of PiZRare genotype among other European countries. Using the Hardy-Weinberg equilibrium method is very difficult and not always accurate to estimate the prevalence of AATD, because different testing methodologies can produce different results. Given the low

At-risk category	Subjects tested (Nr; % of total)	AAT genotypes (Nr confirmed)	Prevalence (% of tested)
COPD	383 (61.77)	23	6.00
Bronchiectasis	239 (38.54)	14	5.85
Emphysema	120 (19.35)	6	5
Asthma	72 (11.61)	5	6.94
Pneumothorax	31 (5.00)	4	12.9
Chronic bronchitis	5 (0.8)	1	20
AATD siblings	8 (1.29)	6	75
Adult cirrhosis	3 (0.48)	1	33.33
Neonate hepatitis	1 (0.16)	1	100

Table 1
DETECTION EFFICIENCY OF ABNORMAL AAT GENOTYPES IN AT-RISK GROUPS OF PATIENTS

Pattern of Genotyping (n=620)	Prevalence (%) of detected genotypes in tested cases (n=620)	Estimated genotypes prevalences based on Hardy – Weinberg equilibrium
PIMM	91.93	1:1.08
PIMS	3.22	1:32
PIMZ	2.58	1:28
PIZZ	0.48	1:2770
PISZ	0.16	1:1000
PIMRare*	1.29	1:48
PIZRare*	0.32	1:12346
PISS	0	1:3906

*Legend: MRare includes the following genotypes: $P_{lowell}M$, IM, $M_{wurzburg}M$.

#ZRare includes the following genotypes: IZ, $P_{lowell}Z$.

Table 2
PREVALENCE OF GENOTYPES DETECTED IN THE ROMANIAN INDIVIDUALS AT RISK (POPULATION TESTED AND ESTIMATED BY HARDY - WEINBERG EQUILIBRIUM)

awareness of an already rare disease, a wide category of pulmonary and gastroenterological indications for testing, both in adults and children, as well as siblings of patients with an AATD genotype were allowed. As the main testing location was a tertiary pulmonary disease hospital in Romania, most screened cases were adults with respiratory pathology (96.6%), while the remaining percentage included children with lung or liver diseases. The most common indication for AATD testing in adults is considered COPD [1,14,17]. In children group, liver disease was the main indication. The proportion of abnormal allele (28.5%) and severe deficit in children (50%) was higher than in adults or data reported by other studies (5-15% of children with liver diseases) [22]. A possible explanation could be that the study was carried out in a tertiary pulmonary center, where children were sent only if they had a high suspicion of AATD. Diagnosis method respected Polish protocol. Sequencing was performed in a limited number of samples (n=33) if a discrepancy between the AAT serum concentration and the genetic test was found. So, it enabled areal identification of rare alleles and explained why actual prevalence of rare mutations was expected to be higher. A number of 45 patients were excluded due to the discrepancies between blood samples with very low AAT plasmatic values and normal genotype. Because of the multitude consecutive samples being in the same situation, this was attributed to the time elapsed between collecting and sending the samples or to the transport conditions. Once the time needed to send the samples improved, discrepancies became very infrequent. However, as the discrepancies appeared at the beginning or the screening, when the sequencing was not easily available, there is a possibility of underestimating the rare cases. 8.1% of tested individuals presented a mutated genotype, lower compare to other studies on at-risk populations, based on decreased serum values, [23–25], but similar to those based on clinical arguments of AATD screening [26], as we did. A number of 3 PiZZ patients (0.5%), including 2 children (with extrapulmonary indications), represented a higher frequency than other research revealed [26]. Estimating detection rates in each at-risk group allowed some considerations. Regarding extrapulmonary categories at-risk, the detection rates are high (100% for neonatal hepatitis, 33.3% for adult cirrhosis combined to respiratory diseases) compare to pulmonary ones. According to classical indications for AATD testing, patients with COPD, asthma, emphysema and bronchiectasis were tested and seemed equally with a detection rate between 5 and 7%. Chronic bronchitis patients were tested for AATD if the most frequent reasons of chronic cough (as COPD, asthma, infectious diseases, otolaryngology pathologies or gastro-esophageal reflux) were excluded. The high detection rate (12.9%) of AATD mutations in spontaneous pneumothorax patients was unexpected but relevant, because this screening indication did not exist in guidelines. However, this is a challenge which should be investigated further, as the specific prevalence is not accurate due to the small cohorts.

This is the first time when AATD is regarded as a medical problem that must be solved in Romania. Many challenges consist in the referrals for genetic testing, as many colleagues used only to check the serum values in AAT testing. This most difficult part of the study, caused by the lack of referrals from gastroenterological clinics, should be resolved in the future by raising AATD awareness among gastroenterologists and by promoting genetic testing where real indication exists, regardless of the AAT concentration. A possible limitation is related to the broad approach of testing both lung and liver diseases, adults and children, index and non-index cases. Considering the goals of this study (feasibility and challenges), it proved that this first study to be the more appropriate approach in order to improve future AATD detection.

Conclusions

This study proves that a targeted DAAT detection program is feasible in Romania. To improve the targeted detection process in the country, a number of six zonal testing centers and coordinators were established. Romania has more rare variants so sequencing must be performed in all high risk cases. A future targeted detection program should extend the testing in both pediatric and adult area. The next important step related to the Romanian detection program will be the implementation of a national AATD registry (in progress).

Acknowledgments and Funding: The analysis of the blood samples was possible due to funding from the project: Dissemination and optimization of alpha-1 antitrypsin deficiency diagnostic algorithm in patients with chronic lung diseases (theme 5/4) of the National Institute of Tuberculosis and Lung Diseases, Warsaw in Poland. The authors are deeply indebted to the Polish Foundation for Patients with alpha-1 antitrypsin deficiency for the support of their collaboration and research activities.

References

1. *** 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 **168**, Nr 7, 2003, p.818.

2. STOLLER, J.K., ABOUSSOUAN, L.S. Am J Respir Crit Care Med **185,**Nr3, 2012, p. 246.
3. DAHL, M, NORDESTGAAR, B.G. Int J Chron Obstruct Pulmon Dis **4**, 2009, p.157.
4. BLANCO, I., de SERRES, F.J., CARCABA, V., LARA, B., FERNANDEZ-BUSTILLO, E. Hepat Mon **12**, 2012, e7434.
5. LAURELL, C-B, ERIKSSON, S. Scand J Clin Lab Investig **15**, Nr 2, 1963 p.132.
6. SVEGER, T. N Engl J Med **294**, Nr 24, 1976, p.1316.
7. LAURELL, C.B., SVEGER, T. Am J Hum Genet **27**, Nr 2, 1975, p. 213.
8. *** Alpha 1-antitrypsin deficiency: MEMORANDUM from a WHO meeting. Bull World Health Organ **75**, Nr 5, 1997, p.397.
9.STOLLER, J.K., BRANTLY, M. J Chronic Obstr Pulm Dis **10**, Suppl. 1, 2013, p.26.
10.GREULICH, T., AVERYANOV, A., BORSA, L., ROZBORILOVA, E., VAICIUS, D., MAJOIR, T, et al. Clin Respir J. 2015, https://doi.org/10.1111/crj.12310
11.LUISETTI, M, SEERSHOLM, N., Thorax **59**, 2004, p. 164.
12. BLANCO, I, de SERRES, F.J., FERNANDEZ-BUSTILLO, E., LARA, B., MIRATVILES, M. Eur Respir J **27**, Nr 1, 2006, p.77.
13. De SERRES, F.J., BLANCO, I, FERNANDEZ- BUSTILLO, E. Monaldi Arch Chest Dis - Pulm Ser. **67**, Nr 4, 2007, p. 184.
14. *** GOLD Global Strategy for the Diagnosis, Management and Prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease 2016. http://goldcopd.org/global-strategy-diagnosis-management-prevention-copd-2016/
15. MARCINIUK, D.D., HERNANDEZ, P., BALTER, M., BOURBEAU, J., CHAPMAN, K.R., FORD, G.T., et al. Can Respir J **19**, Nr 2, 2012, p. 109.
16. VIDAL, R., BLANCO, I., CASAS, F., JARDI, R., MIRATVILES, M., SPANISH SOCIETY OF PULMONOLOGY Guidelines for the Diagnosis and Management of á 1 -Antitrypsin Deficiency, 2006, p.119.
17. SANDHAUS, R.A., TURINO, G., BRANTLY, M.L., CAMPOS, M., CROSS, C.E., GOODMAN, K., et al. COPD: J Chronic Obstr Pulm Dis **3**, Nr 3, 2016, p.668.
18. SERAPINAS, D., OBRIKYTE, V., VAICIUS, D., BALCIUVIENE, R., VALAVICIUS, A., SAKALAUSKAS, R. Pneumologia 2014, p. 32.
19. CHOROTOWSKA- WYNIMKO, J., STRUNIAWSKI, R., SLIWINSKI, P., WAJDA, B., CZAIJKOWSKA-MALINOWSKA, M. COPD: J Chronic Obstr Pulm Dis **12**, Supl 1, 2015, p.22.
20. STRUNIAWSKI, R., SZPECHCINSKI, A., POPLAWSKA, B., SKRONSKI, M., CHOROTOWSKA-WYNIMKO, J. Adv Exp Med Biol **756**, 2013, p.29.
21. CHOROTOWSKA-WYNIMKO, J., STRUNIAWSKI, R., POPLAWSKA, B, BORSZEWSKA-KORNACKA, M. Pneumonol. Alergol. Pol. 2012. p. 450.
22. MOYER, V., FREESE, D.K., WHITINGTON, P.F., OLSON, A.D., BREWER, F., COLLETTI, R.B., et al. J Pediatr Gastroenterol Nutr **39**, Nr 2, 2004, p.115.
23. LUISETTI, M., MASSI, G., MASSOBRIO, M., GURRACI, P., MENCHICCHI, F.M., BECCARIA, M., et al. Respir Med. **93**, Nr 3, 1999, p. 169.
24. GREULICH, T., NELL, C., HERR, C., VOGELMEIER, C,, KOTKE, V., WIEDMANN, S, et al. Orphanet J Rare Dis **11**, Nr 1, 2016, p. 75.
25. CARROLL, T.P., O'CONNOR, C.A., FLOYD, O, McPARTLIN, J, KELLEHER, D.P., O'BRIEN, G, et al. BioMed Central, **12**, Nr 1, 2011, p. 91.
26. SITKAUSKIENE B, SERAPINAS, D., BLANCO, I., FERNANDEZ-BUSTILLO, E., JANCIAUSKIENE, S., SAKALAUSKAS, R., et al. Respir Med **102**, Nr 11, 2008, p. 1654.

Manuscript received: 10.01.2018