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# Population Genetic Screening for Alpha1-Antitrypsin Deficiency in a High-Prevalence Area

Luciano Corda[a] Daniela Medicina[b] Giuseppe Emanuele La Piana[a] Enrica Bertella[a] Giovanni Moretti[c] Luca Bianchi[e] Valentina Pinelli[a] Gianfranco Savoldi[d] Paola Baiardi[f] Fabio Facchetti[b] Nuccia Gatta[g] Isabella Annesi-Maesano[i] Bruno Balbi[h]

[a]Centro di Riferimento Regionale per il Deficit di Alfa1-Antitripsina, Prima Divisione di Medicina Interna, Spedali Civili, Cattedra di Malattie dell'Apparato Respiratorio, Università di Brescia, [b]Primo Servizio di Anatomia Patologica, Spedali Civili, Università di Brescia, [c]Laboratorio di Analisi Chimico Cliniche, Ospedale di Gardone VT, Spedali Civili, [d]Laboratorio di Genetica Pediatrica, Istituto di Medicina Molecolare A. Nocivelli, Spedali Civili, Brescia, [e]Divisione di Pneumologia Riabilitativa, Fondazione Salvatore Maugeri, I.R.C.C.S., Lumezzane, [f]Consorzio per le Valutazioni Biologiche e Farmacologiche, Pavia, [g]Associazione Nazionale ALFA1-AT, Sarezzo, [h]Divisione di Pneumologia Riabilitativa, Fondazione Salvatore Maugeri, I.R.C.C.S., Veruno, Italy; [i]EPAR, U707 INSERM, Paris and EPAR, UMR-S707 UPMC, St. Antoine Medical School, Paris, France



**Key Words**

α1-Antitrypsin deficiency · Population genetic screening

**Abstract**

***Background:*** Current guidelines for α1-antitrypsin deficiency (AATD) state that adult population screening should only be done in high-risk areas. Up-to-date genetic methods are always recommended. ***Objectives:*** To determine the prevalence of AATD in a suspected high-risk area by population screening, applying new genetic analyses and comparing the prevalence of liver and lung abnormalities in subjects with or without AATD. ***Methods:*** Adult residents of Pezzaze, a village in an Italian alpine valley, voluntarily participated in the screening, and were examined for: nephelometric α1-antitrypsin (AAT) serum level, DNA analysis (mutagenic polymerase chain reaction and restriction fragment length polymorphism tests for Z and S AATD causative mutations, and denaturing high-performance liquid chromatography and/or direct gene sequencing if needed), serum aspartate and alanine transaminases, a respiratory questionnaire and the Medical Research Council dyspnea index scale. The prevalence of AATD was compared with that expected in Italy (Hardy-Weinberg equilibrium), and transaminases and the prevalence of respiratory symptoms were compared between study groups. ***Results:*** Of 1,353 residents, 817 (60.4%) participated; 67 (8.2%) had low AAT serum levels (<90 mg/dl); 118 were carriers of AATD-associated alleles, 4 (0.5%) homozygotes or compound heterozygotes (1 Z, 1 S, 2 $ZP_{brescia}$), 114 (14%) heterozygotes (46 Z, 52 S, 9 $P_{brescia}$, 4 $M_{wurzburg}$, 2 I, 1 $P_{lowell}$). The prevalence and frequency of all AATD-related alleles was higher than expected for Italy ($p < 0.001$). There were no differences in symptoms of respiratory disease and transaminases between individuals with normal and low

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Luciano Corda, MD
Prima Divisione di Medicina, Spedali Civili
P.le Spedali Civili 1
IT–25123 Brescia (Italy)
Tel. +39 03 0399 5237, E-Mail luciano.corda@spedalicivili.brescia.it

serum AAT. ***Conclusion:*** The screening design is one of the main strengths of this study. The large number of mostly asymptomatic individuals with AATD identified suggests that in high-risk areas adult population screening programs employing the latest genetic methods are feasible. Early recognition of individuals at risk means primary or secondary prevention measures can be taken.



## Introduction

α1-antitrypsin deficiency (AATD) is a genetic codominant condition characterized by low plasma levels of α1-antitrypsin (AAT). Although related to other clinical disorders and representative of a syndrome, AATD tends to lead to lung and liver diseases and can be devastating for many patients, as reflected in high rates of liver and lung transplantation [1–5].

AAT is a pleomorphic glycoprotein with at least 100 identified variants [6]; normal variants are referred to as M. Approximately 30 mutations are reported to reduce AAT plasma levels [7], Z and S being the two most frequent; other variants are rare, some of them only described as single case reports.

Although the clinical manifestations of AATD are considered rare disorders, it has been recently estimated that AATD is one of the most common serious hereditary disorders in the world [3]. This is due to: (1) incomplete penetrance, also related to gene-environment interactions (such as smoking); (2) lack of specific phenotypes associated with lung and liver AATD; (3) scant awareness of the condition; (4) scarcity of screening programs [8].

Population screening for AATD is a controversial issue [9]. A recent document by the American Thoracic Society (ATS) and the European Respiratory Society (ERS) recommended adult population screening in areas where there is a suspected high prevalence of AATD [6]. The most recent genetic techniques are always recommended [10]. The records of a hospital-based AATD case-finding program in Italy [11] suggested a high prevalence of AATD in a population whose families originated from some villages in an Italian alpine valley (Val Trompia), north of Brescia (Lombardy). The aim of this study was therefore to determine the prevalence of AATD in a suspected high-risk area by population screening for AATD applying new genetic analyses and comparing the prevalence of liver and lung abnormalities in subjects with or without AATD.

## Methods

The study was an adult population screening for AATD on a voluntary basis. The study protocol was approved by the local health authority ethics committee. Exclusion criteria were unwillingness to take part in the screening and inability to understand the information leaflet.

Blood samples were taken from residents who signed the consent forms. Serum AAT was assayed using an immune-nephelometric method (Dade Behring, Deerfield, Ill., USA; normal range 90–200 mg/dl). Genetic screening for Z and S AATD mutations was done using polymerase chain reaction (PCR)-mediated site-directed mutagenesis followed by restriction fragment length polymorphism analysis. DNA was isolated from peripheral whole blood with a standard salting-out protocol [12]. The procedure for mutation analysis was taken from the method described elsewhere [13, 14]. Each set of samples was analyzed along with a no-template control, a negative control (wild-type individual) and a positive control (SZ patient). All tests were run twice, at different times by two separate operators, to minimize the risk of contamination.

From a preliminary analysis of the results (fig. 1), subjects negative for Z and S mutations with AAT serum levels between 40 and 105 mg/dl were considered at risk of having at least one non-Z non-S allele associated with AATD. We also considered inconsistent any result indicating heterozygosity for either Z or S mutation in subjects with serum AAT between 40 and 90 mg/dl (90 mg/dl being the instrument cutoff of the immune-nephelometric method). The samples from these two subgroups were analyzed by denaturing high-performace liquid chromatography (DHPLC). Coding and flanking regions of exons II, III and V of the AAT gene were analyzed; mutational analysis was done with the Transgenomic WAVE instrument (Transgenomic, Omaha, Nebr., USA). DHPLC is based on the principle of separation of the homo- and heteroduplex formation of individual PCR products at specific temperatures and set gradients. Once the chromatographic fingerprints were established for each exon of the AAT gene, the PCR products were analyzed at partially denaturing temperatures to detect DNA changes. DHPLC is considered a highly sensitive method (>95%) [15].

Samples showing mutations in DHPLC (abnormal peaks) and DNA from subjects heterozygous for either a Z or an S mutation and serum AAT <40 mg/dl were sequenced (fig. 1). Exons II, III and V of the selected samples were amplified by PCR and analyzed by automated fluorescent sequencing using the Big-Dye-Terminator-Kit 3.1 and an ABI3130 sequencer (Applied Biosystems, Foster City, Calif., USA). Aspartate and alanine transaminases in serum were assayed enzymatically [Olympus, High Wycombe, UK; normal ranges: aspartate transaminases 0–31 U/l (female), 0–35 U/l (male); alanine transaminases 0–34 U/l (female), 0–45 U/l (male)].

All individuals completed the European Community Health Respiratory Survey (ECRHS) questionnaire [16], to detect symptoms of rhinitis, asthma, or chronic bronchitis. We also recorded the Medical Research Council (MRC) dyspnea index for each person [17].

A letter was sent to every resident in Pezzaze, asking for participation in the study. They were preliminarily informed about the study by pamphlets and public meetings. Those who took part were notified of their results by letter, and clinical and genetic counseling was offered for people with low AAT levels and abnormal genetic results.

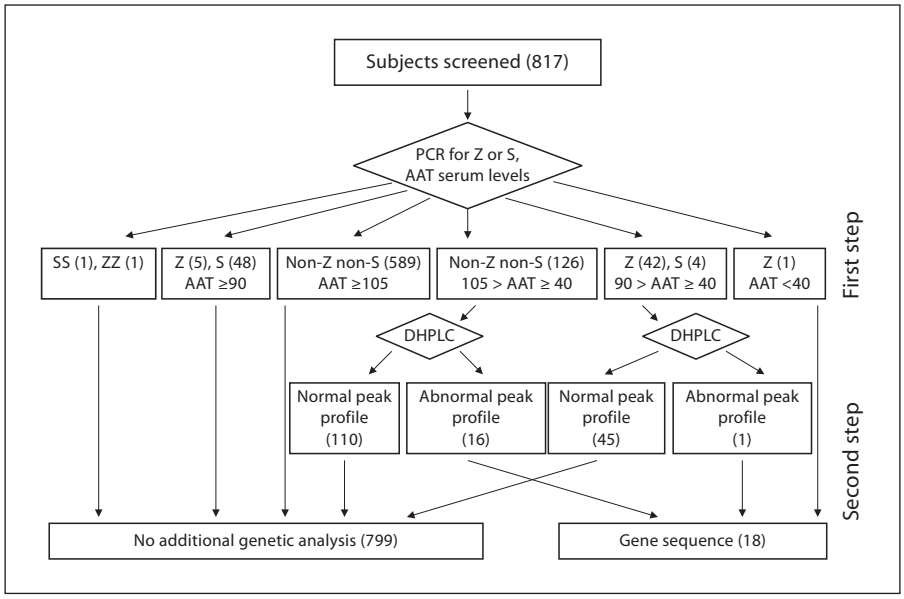


**Fig. 1.** Algorithm and results for the identification of the various genotypes and distribution of the 817 subjects screened, according to their α1-antitrypsin genotype. AAT serum levels are expressed as mg/dl.

*Statistical Analysis*

Data were analyzed with the GraphPad Prism Version 4 (GraphPad Software Inc., San Diego, Calif., USA) and with SAS for Windows V 9.1 (SAS, Cary, N.C., USA). Subjects' characteristics were reported using descriptive statistics and expressed as means ± SD. Allelic frequencies were calculated by considering one allele couple for each person screened. Distribution of diagnosed alleles was compared with the expected distribution as obtained by Hardy-Weinberg analysis [3] using the $\chi^2$ test. An unpaired t test was used when appropriate. Means were compared using ANOVA and percentages by the $\chi^2$ test. A p value <0.05 was considered significant.

## Results

A total of 1,353 residents of Pezzaze (708 males and 645 females, mean age 48 ± 17 years) were 18 years of age or older as of 1 April, 2005. Serum and DNA samples were obtained from the 817 people who volunteered to participate (400 males, 417 females, mean age 50 ± 17 years). They constituted 60.4% of the total residents. The proportion of females among the participants was higher than in the total adult population ($p < 0.05$).

Serum AAT assays identified two subpopulations: those with normal levels (≥90 mg/dl) and those with low levels (<90 mg/dl). In total, 67 subjects (8.2% of the total screened population) had low serum AAT. In this subpopulation, there were more males than females than in the total screened population (49.0% of the males in the total screened population vs. 65.7% of the males in subjects with low serum AAT, $p < 0.05$).

**Table 1.** Demographic and AAT genotype data in the subjects with normal and low serum level

| | Normal individuals (serum AAT ≥90 mg/dl) | AATD individuals (serum AAT <90 mg/dl) |
|---|---|---|
| Subjects | 750 | 67 |
| Male/female | 356/394 | 44/23 |
| Male, % | 47.5 | 65.7* |
| Mean age ± SD, years | 50 ± 16.8 | 49 ± 15.5 |
| Genotype | | |
| MM | 692 | 7 |
| MS | 48 | 4 |
| MZ | 5 | 41 |
| $MP_{brescia}$ | 3 | 6 |
| $MM_{wurzburg}$ | 1 | 3 |
| $ZP_{brescia}$ | 0 | 2 |
| MI | 1 | 1 |
| $MP_{lowell}$ | 0 | 1 |
| SS | 0 | 1 |
| ZZ | 0 | 1 |

* $p < 0.5$ versus normal individuals.

PCR analysis indicated that of the 817 subjects tested, 1 (0.1%) was Z homozygote, 1 (0.1%) S homozygote, 52 (6.4%) S-heterozygotes and 48 (5.9%) Z heterozygotes. Thus, PCR detected 102 people (12.5% of those screened) carrying AATD-related alleles.

We identified three subgroups with AAT levels inconsistent with the results of PCR screening for Z and S mu-

**Table 2.** Hardy-Weinberg equilibrium of allele frequency and the number and percentages of different categories related to AATD genes in estimates for the Italian population and observed in the population screened here

| Alleles | Observed | | Expected | |
|---|---|---|---|---|
| | number | frequency | number | frequency |
| M | 1,512 | 0.9253 | 1,570 | 0.9616 |
| S | 54 | 0.0318 | 39 | 0.0226 |
| Z | 50 | 0.0301 | 23 | 0.0143 |
| Rare | 18 | 0.0098 | 2 | 0.0015 |
| Total | 1,634 | 1 | 1,634 | 1 |
| **Genotypes** | **Observed** | | **Expected** | |
| | number | % | number | % |
| Normal subjects | | | | |
| MM | 699 | 85.5 | 755 | 92.5 |
| Heterozygotes | | | | |
| MZ, MS, Mrare | 114 | 14 | 61 | 7.4 |
| Homozygotes or compound heterozygotes | | | | |
| ZZ, SS, Zrare | 4 | 0.5 | 1 | 0.1 |
| Total | 817 | 100 | 817 | 100 |

$\chi^2$ test $p < 0.001$ for all comparisons of expected and observed allele frequency. $\chi^2$ test $p < 0.001$ for all comparisons of expected and observed numbers and percentages for different AAT categories.

tations (fig. 1): (1) 126 with no Z or S mutation and serum AAT between 40 and 105 mg/dl; (2) 42 Z heterozygotes; (3) 4 S heterozygotes with serum AAT between 40 and 90 mg/dl. DNA from these three subgroups was analyzed by DHPLC as a second step (fig. 1) and 17 samples with abnormal peaks underwent sequence analysis. DNA from a subject with a Z-heterozygote mutation and AAT <40 mg/dl was sequenced directly (fig. 1).

Through this procedure, 18 additional AATD-related variants were detected: 2 subjects (0.2%) were compound heterozygotes for the two mutations Z and $P_{brescia}$ [18, 19], 9 (1.1%) were $P_{brescia}$ heterozygotes, 4 (0.5%) $M_{wurzburg}$ [20] heterozygotes, 2 (0.2%) I [21] heterozygotes and 1 (0.1%) $P_{lowell}$ [22] heterozygote (fig. 1; table 1).

On grouping the data from the first PCR analysis with the DHPLC and sequencing findings, we saw that 118 subjects (14.5% of the screened population) had one AATD-related allele (fig. 2). Four (0.5 % of the total, 1 Z-homozygote, 1 S-homozygote and 2 $ZP_{brescia}$ compound heterozygotes) were either homozygotes or compound heterozygotes for AATD mutations. Thus, the prevalence of homozygous or compound heterozygotes AATD in the Pezzaze population was 1/204, while 114 (14%) were simple heterozygotes, giving a prevalence of carriers of AATD-related alleles in this population of 1/7.6.

Previous data [3] show that in Italy, following the Hardy-Weinberg equilibrium, the estimated frequencies of AATD alleles are 0.9616, 0.0226 and 0.0143 for M, S and Z, respectively; as a difference from these estimates (1 – 0.9616 – 0.0226 – 0.0143), we can consider 0.0015 the estimated frequency of AATD rare variants. In this study we observed an S allele frequency of 0.0318, a Z allele frequency of 0.0301 and a rare variants frequency of 0.0098. The $\chi^2$ test indicated that all these frequencies were significantly higher than expected (table 2). A significant difference was also detected between the expected and observed numbers of subjects when grouping all individuals that were homozygous or compound heterozygotes for AATD mutations, heterozygous for AATD mutations and wild type (table 1).

As expected, the majority of Z heterozygotes (89%) had serum AAT <90 mg/dl and the minority (8%) of S-heterozygotes had the same result (table 3). The 7 individuals with an M-homozygous genotype but low serum AAT might be explained by some new, still unknown rare variants.

**Table 3.** Summary of the results for individuals identified as carriers of AATD genes

| | MM | MS | MZ | $MP_{brescia}$ | $MM_{wurzburg}$ | $ZP_{brescia}$ | MI | $MP_{lowell}$ | SS | ZZ |
|---|---|---|---|---|---|---|---|---|---|---|
| No. | 699 | 52 | 46 | 9 | 4 | 2 | 2 | 1 | 1 | 1 |
| Male/female | 350/349 | 16/36 | 23/23 | 6/3 | 2/2 | 0/2 | 1/1 | 0/1 | 1/0 | 1/0 |
| Male, % | 50.1 | 31.0 | 50.0 | 66.7 | 50.0 | 0 | 50.0 | 0 | 100 | 100 |
| Age, years | 50 ± 17 | 47 ± 16 | 52 ± 18 | 54 ± 19 | 49 ± 19 | 50, 54 | 43, 43 | 54 | 40 | 38 |
| Serum AAT, mg/dl | 126 ± 25 | 111 ± 21 | 77 ± 12 | 85 ± 9 | 86 ± 6 | 28, 40 | 86, 96 | 77 | 86 | 23 |
| Range | 79–279 | 78–173 | 61–133 | 71–100 | 79–94 | – | – | – | – | – |
| Subjects with AAT <90 mg/dl | 7 (1%) | 4 (8%) | 41 (89%) | 6 (67%) | 3 (75%) | 2 (100%) | 1 (50%) | 1 (100%) | 1 (100%) | 1 (100%) |

Age and serum AAT are expressed as means ± SD if the group comprises more than two subjects. MM = M homozygotes; MS = S heterozygotes; MZ = Z heterozygotes; $MP_{brescia}$ = $P_{brescia}$ heterozygotes; $MM_{wurzburg}$ = $M_{wurzburg}$ heterozygotes; $ZP_{brescia}$ = $ZP_{brescia}$ compound heterozygotes; MI = I heterozygotes; $MP_{lowell}$ = $P_{lowell}$ heterozygotes; SS = S homozygous; ZZ = Z homozygotes.

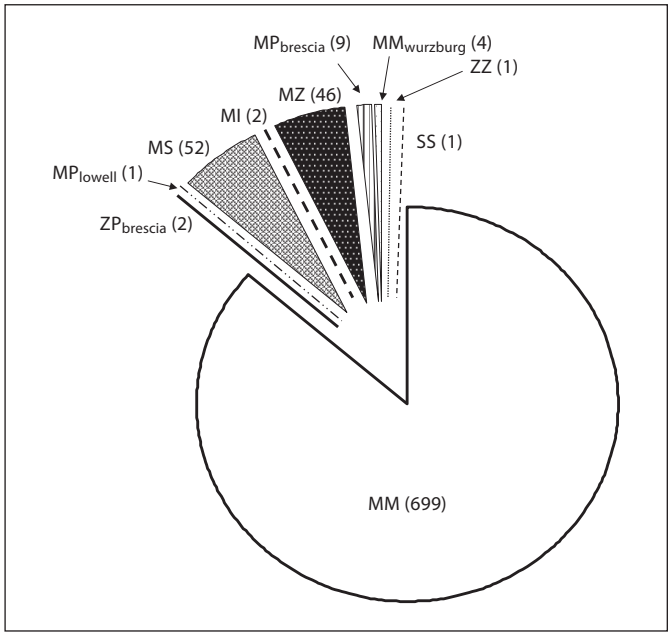


**Fig. 2.** Distribution of the 817 subjects screened, according to their α1-antitrypsin genotype. MM = M homozygotes; $ZP_{brescia}$ = $ZP_{brescia}$ compound heterozygotes; $MP_{lowell}$ = $P_{lowell}$ heterozygotes; MS = S heterozygotes; MI = I heterozygotes; MZ = Z heterozygotes; $MP_{brescia}$ = $P_{brescia}$ heterozygotes; $MM_{wurzburg}$ = $M_{wurzburg}$ heterozygotes; ZZ = Z homozygotes; SS = S homozygotes.

To analyze the clinical relationships with AATD, we also classified the subjects by their serum AAT levels, as this is considered a critical factor. There were no significant differences between individuals with normal and those with low serum AAT for any of the symptoms identified by the ECRHS questionnaire or for the MRC dyspnea index (table 4). However, on grouping individuals by their AAT genotype we found a significant difference between normal individuals (M homozygotes) and heterozygotes, the former reporting more symptoms of chronic bronchitis than the simple heterozygotes (Z heterozygotes, S heterozygotes and rare genotype heterozygotes) (13.5 vs. 6.1% of the rest of the population, $p < 0.05$). This difference was not related to differences in age, sex, smoking or occupational history. No differences were seen in any other symptom including dyspnea between M-homozygous individuals and heterozygotes. There were also no differences in any respiratory symptom or in dyspnea between the three heterozygous categories.

Serum transaminases were also comparable in individuals with normal or low AAT levels. Similarly, when grouping individuals by the presence or absence of AATD-related alleles, 16 of 118 individuals with (13.6%) and 92 of 669 without AATD alleles (13.8%) had abnormal transaminases (not significant). Serum transaminases were high in 7/52 S heterozygotes, 8/46 Z heterozygotes and 0/15 rare genotype heterozygotes.

## Discussion

The main findings of this study are: (1) the use of an innovative diagnostic algorithm to analyze samples collected in screening programmes; (2) the finding of a higher than expected prevalence of AATD in a study population; (3) the identification of a considerable number of rare mutations of AATD; (4) the absence of clinical relationships with lung or liver disease in the majority of subjects with low serum levels of AAT.

**Table 4.** Prevalence of allergic and respiratory symptoms detected by the ECRHS questionnaire

| Symptoms | Total population | Normal individuals (serum AAT ≥90 mg/dl) | AATD individuals (serum AAT <90 mg/dl) |
|---|---|---|---|
| Allergic rhinitis | 11.0 | 11.1 | 9.9 |
| History of asthma | 7.7 | 7.7 | 7.0 |
| Diagnosis of asthma | 6.1 | 6.3 | 4.4 |
| Chronic bronchitis | 12.5 | 13.0 | 7.0 |
| Dyspnea | 11.0 | 11.5 | 5.8 |

Figures are percentages. No significant difference for any comparison between groups.

Concern about the potential psychological, social and economic effects on study subjects and the probably very low yield of systematic adult population screening led the ATS and ERS experts to discourage it for AATD [6], except in certain circumstances such as a high prevalence of AATD (>1/1,500) and high smoking rates. It was also recommended that semi-automated genetic techniques [10] be employed for population screening for AATD in probable high-prevalence areas. Considering the high proportion of smokers in Italy, our study satisfies all these conditions.

On the basis of recent estimates [3], we would have expected to detect approximately 61 persons of the 817 studied carrying one AATD allele (that is, heterozygotes) and one carrying both alleles with AATD (that is, homozygotes or compound heterozygotes). However, we found 118 carrying one AATD allele, and 4 with severe deficiency. Thus, the prevalence of each AATD allele (Z, S and rare variants) was much higher than recently estimated for Italy [3], even considering the breakdown of epidemiological data from different Italian regions, including the northern Italian regions close to Lombardy [23]. This can certainly be considered a high prevalence, as it is far beyond the limit of 1/1,500 subjects mentioned in the ATS/ERS statement, meaning that the project was justified by the screening results.

The screening design is one of the main strengths of this study. We applied innovative genetic techniques to develop a specific algorithm for genetic testing and genotyping of samples. The method involved a graded process of screening and required direct sequencing on a limited number of samples (only 18), 17 of which had an abnormal DHPLC peak profile and all of them were carriers of a rare AATD causative variant. This shows that DHPLC can distinguish normal from pathological alleles and can be useful for screening large numbers of samples when rare alleles are suspected [24] or where there are known to be large numbers of rare alleles, such as in Italy [25]. Claims about the sensitivity of DHPLC vary but largely reflect the users' expertise, the care taken in designing the analysis and the sequence examined. Our instrument settings (size of fragments analyzed and number of injection temperatures used) gave high sensitivity.

The algorithm employed might also be useful for other screening programs or in other AATD diagnostic approaches, since it provides the reasonable accuracy needed in testing samples for genetic conditions. Similar algorithms have been proposed to identify people at risk for AATD [26, 27].

Together with a higher prevalence of Z and S alleles, we also observed a high frequency of rare alleles. The prevalence of rare AAT variants in Italy is the highest recorded worldwide so far, though, interestingly, the country has a medium-low prevalence of the common ZZ genotype [25]. This demonstrates the importance of genetic screening for AATD in high-risk areas, as it permits the identification of rarely identified variants. In this context, we found 11 individuals with the $P_{brescia}$ allele. This is a novel mutation characterized by a Gly/Arg substitution at position 225, due to a point mutation on exon III (c.745C>G) that we identified [18, 19].

The transaminase levels and respiratory symptoms in the groups (divided on the basis of AAT serum levels or genotype), and the lack of respiratory symptoms in 3 of the 4 severe AATD patients suggests screening is an effective preventive medical intervention. The apparent discrepancy in increased respiratory symptoms among individuals without AATD alleles (M homozygotes) compared with heterozygous subjects might be due to selection bias, since patients with respiratory symptoms may well be more likely to be tested. The lack of a clinical relationship for lung diseases in the subjects with low serum levels might be due either to the assessment method or to the age of the participants, or both.

The position of the village, in a small, closed-in alpine valley (Val Trompia), and the low levels of genetic mixing also help explain these results. In addition, tobacco smoking is highly prevalent among the local adults and until the early 1980s there was a mining company in the area. Our results suggest that population screening for AATD might be useful in other high-risk areas too. This concept is important from an epidemiological point of view, as

confined areas and populations with a prevalence of AATD are a feature of many countries, as recently demonstrated for the island of Madeira (Portugal) [28]. The screening of the population in those areas would allow early diagnosis of AATD-related diseases or the identification of asymptomatic AATD individuals and would make it possible to apply prevention measures, both primary (such as smoking cessation and alcohol abstention campaigns) and secondary (treatment of related lung or liver diseases) and to extend the test for AATD to first-degree relatives of identified carriers [29]. All these aspects can be considered indicators of the utility of screening.

This study has some limitations. The population size and the difference between the sex distribution of the total residents and the screened population can be considered a 'side effect' of our policy of screening adult volunteers, but we cannot rule out the possibility that in a population more representative of the whole resident population the results could be different. However, we do believe that the findings are valuable despite this limitation, which is inevitable in screenings run on a voluntary basis. We used the ECRHS questionnaire (which collects information on symptoms and exposure to risk factors for asthma) and the MRC dyspnea test (which is a measure of perceived breathlessness) to assess respiratory function during the screening; admittedly, this method does not always allow the objective detection of respiratory failure. Another limit might be the fact that we did not record the emotional impact of the screening among the people who took part. No systematic cost-efficacy analysis was done, as this was not included in the endpoints of the study.

In conclusion, the large number of mostly asymptomatic individuals identified as having AATD, a genetic condition potentially leading to fatal lung or liver diseases, suggests that in high-risk areas adult population screening program employing up-to-date genetic methods may be useful. Early recognition of individuals at risk offers the opportunity to apply primary or secondary prevention measures.


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