

Screening for α_1 -Pi deficiency in patients with lung diseases

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ABSTRACT: In patients with pulmonary emphysema, studies have reported 2–3% of individuals with severe α_1 -Pi deficiency. The aims of this study were to evaluate the accuracy of a new method for quantifying α_1 -Pi through phenotyping from dried blood spots (DBS) and to test the hypothesis that the screening of a population at risk increases the detection rate for severe α_1 -Pi deficiency.

The accuracy of phenotyping results from DBS was compared to conventional methods in a total of 555 individuals. In a prospective study 1,060 patients with chronic lung disease were screened for α_1 -Pi deficiency using DBS.

The validation of the phenotyping method from DBS showed an accuracy of 100%. Out of 1,060 tested patients, none had a severe PiZ deficiency and only 3 had PiSZ, whilst 36 (3.34%) individuals were identified as heterozygous for PiMS and 39 (3.68%) for PiMZ.

No patients with severe α_1 -Pi deficiency could be detected in this population and the frequency of PiMS or PiMZ detected was similar to that of the normal population. Thus, the screening of an unselected population of chronic obstructive pulmonary disease and asthma patients may not detect a large number of individuals with severe α_1 -Pi deficiency.

Eur Respir J 2002; 20: 319–324.

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Keywords: α_1 -Pi deficiency asthma, chronic obstructive pulmonary disease, gene frequency, screening

Received: October 27 2001

Accepted after revision: March 11 2002

The study was supported by a grant from Bayer Corp., Research Triangle Park, NC, USA. E.J. Campbell's work was supported by PHS grant HL 46440.

Patients with severe α_1 -Pi deficiency suffer from increased morbidity and mortality, which is mainly due to the development of chronic obstructive pulmonary disease (COPD) and pulmonary emphysema [1, 2]. The major known risk factors are active and passive smoking. Patients who stop smoking have a better prognosis than those who do not [3]. Thus, an early detection of α_1 -Pi deficiency would enable patients to make lifestyle changes to either prevent or postpone the development of functional impairment. However, in most European countries and the USA, <10% of the expected homozygous PiZ individuals are identified [4–6]. Implementing effective screening efforts could increase the rate of detection and enable early preventive measures to be taken.

Several studies found between 1.9–17.8% of homozygous PiZ individuals in referral groups of patients with COPD [7–9] indicating that a targeted screening of patients with COPD could significantly increase the detection of homozygous PiZ individuals. On this basis, the World Health Organization (WHO) recommended that all patients with COPD and adults and adolescents with asthma should be screened once in their life for α_1 -Pi deficiency using a quantitative test [10].

The diagnosis of α_1 -Pi deficiency is based on the measurement of α_1 -Pi serum levels and isoelectric focusing (IEF). So far, large screening efforts for α_1 -Pi

deficiency have been hampered by the need to draw intravenous blood and send it to the testing laboratory for both types of laboratory tests. Using dried blood spots (DBS) both for quantitative measurements of α_1 -Pi and for determination of the phenotype would obviate the need for refrigeration and simplify handling and shipping. However, the use of DBS in large-scale screening programmes has not been validated. Therefore, the aims of this study were: 1) to determine the accuracy of the new DBS test for α_1 -Pi concentrations and phenotyping and to assess the feasibility of using DBS in physicians' offices for screening purposes; 2) to evaluate the gene frequencies of deficiency alleles in a large population of patients with chronic lung disease; and 3) to determine whether applying the WHO recommendations for the screening of patients with COPD and asthma can increase the detection of patients with severe α_1 -Pi deficiency, an important prerequisite for large-scale screening of a target population.

Methods

Validation of testing for α_1 -Pi deficiency using dried blood spots

The processes for using DBS for the quantitative immunoassay and IEF were preliminarily evaluated

with regard to sensitivity and specificity. For detection of α_1 -Pi deficiency, aliquots of 427 samples of ethylenediamine tetracetic acid (EDTA)-anticoagulated whole blood were allowed to dry onto filter paper. Both the EDTA-anticoagulated blood and the dried blood were then subjected to α_1 -Pi immunoassay as described below. Dried blood samples that yielded α_1 -Pi concentrations $<20 \mu\text{M}$ and the respective plasma samples were further subjected to IEF.

Field test

For subsequent direct validation of the immunoassay and phenotyping procedures, individuals who attended a National Educational Conference of the Alpha-1 Association (Boston, MA, USA) (a population expected to include many individuals with α_1 -Pi deficiency) were offered an opportunity to simultaneously donate EDTA-anticoagulated whole blood obtained by phlebotomy and blood obtained by puncture of a distal finger pad for drying onto filter paper. A total of 128 subjects provided informed consent and participated in the study. The samples were analysed by immuno assay as described below. All DBS samples and the corresponding whole blood samples were phenotyped.

Testing for α_1 -Pi deficiency in selected practitioners' offices in Germany

A total of 7 physicians' offices took part in the screening. Four were general practitioners and three were pulmonary physicians. One of them treated a single patient with α_1 -Pi deficiency. Between March and June of 1999, patients with either COPD (chronic bronchitis, chronic obstructive bronchitis and pulmonary emphysema), asthma, or bronchiectasis were offered a test for α_1 -Pi concentrations and phenotype. Written informed consent was obtained and blood samples were collected on filter paper for further processing. Attached to the filter paper was a questionnaire on which physicians ticked boxes relating to smoking history, clinical symptoms and pulmonary diagnosis.

A total of 1,156 samples were received for evaluation. In 89 cases poor spotting of the blood on the filter paper prevented detection of both α_1 -Pi concentrations and phenotyping. In seven cases, there was enough material for the determination α_1 -Pi concentrations only. Thus, 1,060 cases were available for further analysis.

Application of blood onto filter paper and subsequent elution

Whole blood, either obtained from a distal finger pad with a lancet, a hyperemic earlobe, or an EDTA-anticoagulated blood specimen obtained by phlebotomy, was spotted onto no. 903 paper (Schleicher & Scheull, Keene, NH, USA) to completely saturate circles of half an inch in diameter imprinted on the

paper. The papers were then air-dried at room temperature and stored at 4°C in separate envelopes to avoid cross contamination. Samples were shipped in batches once a week *via* overnight courier to the laboratory. For subsequent analysis, 1/8-inch diameter aliquots of paper containing dried blood were either punched from the filter paper by hand or were punched in duplicate directly into microtiter plates using a Delphia Plate Punch (Wallac Oy, Turku, Finland).

Immunoassay for α_1 -Pi

Completely automated immunoassays for α_1 -Pi in EDTA-anticoagulated blood were performed in multi-well plates, as described previously [11]. For the immunoassay of blood dried onto filter paper, α_1 -Pi was eluted from the filter paper aliquots using a Tecan RSP 8051 ID robotic sample processor (Tecan US, Durham, NC, USA), using 0.01 M phosphate, 0.9 M NaCl, 0.05% Tween, $0.1 \text{ mg}\cdot\text{mL}^{-1}$ bovine serum albumin, 2.5% glycerol, pH 7.4 for 60 min, then diluted 1:17 into 96-well Fluoricon plates (IDEXX, Inc., Westbrook, ME, USA). The final multiwell plate included the following, each in duplicate: 1) a reference sample, 2) a normal control sample, 3) a sample from an individual known to have α_1 -Pi deficiency (PiZ), and 4) blank wells and four standards of known α_1 -antitrypsin concentration.

A completely automated particle-concentration immunoassay was then performed in a Screen Machine (IDEXX), essentially as described previously [11]. Digital fluorescence data were transferred to microcomputers, which referenced the unknown samples to the standards and generated reports of the results.

Isoelectric focusing

For determination of α_1 -Pi phenotypes, samples of EDTA-anticoagulated whole blood were subjected to IEF in polyacrylamide gels (LKB Multiphor II and LKB Macrodrive 5 Constant Power Supply, Amersham Pharmacia Biotech, Piscataway, NJ, USA), essentially as previously described [12].

The α_1 -Pi phenotypes of samples of dried blood were determined by adding dithiothreitol to the eluted blood to a final concentration of 0.5 M , then subjecting the sample to IEF. Following IEF, proteins were passively transferred to nitrocellulose paper and incubated with rabbit α_1 -Pi, then with goat anti-human immunoglobulin (Ig)G (Sigma-Aldrich, St Louis, MO, USA). The α_1 -Pi was then visualised by staining with nitro blue tetrazolium [13].

Statistical methods

Data are presented as mean \pm SD. To compare differences between groups, a one way analysis of variance (ANOVA) was used. The Friedman test was used for variables that were not normally distributed.

A Mann-Whitney test was performed for the comparison of two unrelated samples. In the analysis of the influence of different phenotypes on the α_1 -Pi concentration, the nonparametric Kruskal-Wallis H test was used. To assess the relationship of two categorical variables, the Chi-squared test was used. The Pearson's coefficient was calculated to analyse the linear correlation of two variables.

Results

Validation of testing using dried blood spots

A comparison of the immunoassay results obtained from 427 DBS samples with the respective plasma samples are shown in table 1. The agreement between immunoassay results obtained from these two samples was excellent. Clinically significant α_1 -Pi deficiency is characterised by an α_1 -Pi serum concentration of <11 μ M. All samples having α_1 -Pi concentrations <11 μ M with the plasma immunoassay also had α_1 -Pi concentrations <11 μ M with the immunoassay from the corresponding dried blood sample.

When the DBS samples with α_1 -Pi concentrations <20 μ mol were phenotyped, all 29 individuals with levels <11 μ M, but no others, had the deficient phenotype PiZ. Thus, the samples dried onto filter paper yielded no false-positives or false-negatives for α_1 -Pi deficiency, and all phenotypes determined from dried blood were identical to those determined from liquid blood.

Field study

Results of α_1 -Pi immunoassays of the 128 simultaneously drawn paired liquid and dried blood samples obtained at the National Educational Conference of the α_1 National Alpha-1 Association are shown in figure 1.

The correlation coefficient between plasma and DBS α_1 -Pi concentrations was 0.951 ($p < 0.0001$). All of the DBS samples with α_1 -Pi levels <11 μ M, but no others, had α_1 -Pi concentrations <11 μ M by standard immunoassay of liquid venous blood (table 1).

Individuals with an α_1 -Pi <11 μ M, but no others, were found to have phenotype PiZ. In total there were 52 M, 7 MS, 36 MZ, 2 SZ and 26 Z individuals. Four

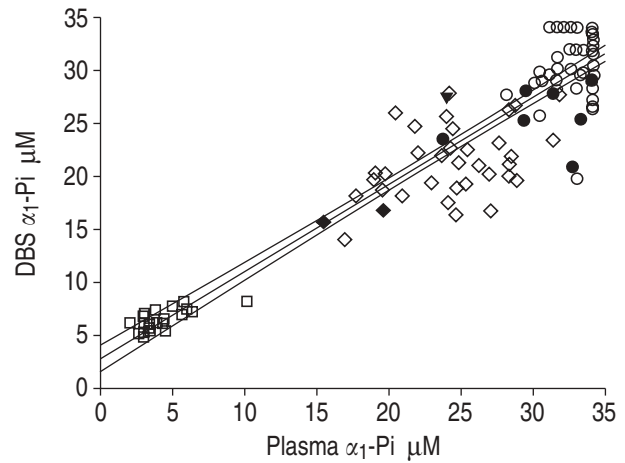


Fig. 1.—Concentrations of α_1 -Pi obtained by finger prick in comparison with intravenous blood samples from the same individuals stratified for their phenotype: \blacktriangledown : prolactin; \square : PiZ; \bullet : PiMS; \circ : PiM; \blacklozenge : PiSZ; \diamond : PiMZ. DBS: dried blood spot.

subjects had the combination of an M allele and suspected rare allele on both phenotyping tests. One patient was on intravenous augmentation therapy with α_1 -Pi, and therefore no attempt was made to phenotype this individual. All phenotyping results on DBS were concordant with the corresponding venous blood samples.

Demographics of the screening population in Germany

A total of 1,060 individuals diagnosed with lung disease visiting a physician's office were screened for α_1 -Pi deficiency. Seventy-seven per cent of the patients (819 cases) were screened by pulmonary physicians; the remaining 241 were seen by general practitioners.

Four-hundred and ninety-eight (47%) were male and 548 (51.7%) were female. No information on sex was given in 14 individuals (1.2%). The mean age for the study group was 52.5 ± 18.9 yrs (median 56.4 yrs, range 5–91 yrs). Eighty-seven individuals were <18 yrs and 217 were >70 yrs. Self-reported smoking history revealed 22.5% smokers, 33.0% ex-smokers, and 38.1% nonsmokers. Information was missing in the remaining 68 patients (6.4%). The mean age was higher in ex-smokers compared to nonsmokers and

Table 1.—Comparison of α_1 -Pi concentrations measured in plasma and dried blood spots

α -Pi concentration in plasma μ M	α -Pi concentration in dried blood spots μ M							Total
	<11	11–15	16–20	21–25	26–30	31–34	>34	
>34					4	15	108	127
31–34				4	13	30	92	139
26–30			1	13	33	13	30	90
21–25			3	15	12	2	2	34
16–20		1	3	2	1			7
11–15			1					1
<11	29							29
Total	29	1	8	34	63	60	232	427

Table 2. – Frequencies of diagnosis

Diagnosis	Frequency	%
Asthma	419	39.5
COPD	390	36.8
Emphysema	301	28.4
Bronchiectasis	17	1.6
Others	158	14.9
Chronic bronchitis	75	7.1
Acute bronchitis	32	3.0
No information	45	4.2

Since some patients were diagnosed with more than one disease, the sum of the percentages is >100. COPD: chronic obstructive pulmonary disease.

smokers (60.7±14.7 yrs *versus* 47.6 and 49.8 yrs, respectively, $p<0.0001$).

Dyspnoea on exertion was the most frequent complaint (69.3%), followed by cough (61.9%), phlegm (50%), asthma attacks (43.3%), and wheezing (42.1%). Only one-fifth of the patients presented with a single complaint, 22.4% had two symptoms that were listed on the questionnaire, 17.4% had three, 12.6% had four, and 27.1% had all five symptoms.

Asthma was the most frequent diagnosis made by the treating physicians in these patients and was present in 419 patients (table 2). In 89.9%, asthma was not accompanied by other diseases. As expected, asthma was the most frequent diagnosis in adolescents with a prevalence of 78% in the population tested. COPD was diagnosed in 390 patients, and in almost half of the cases this diagnosis was associated with other lung diseases. Pulmonary emphysema was found in 301 patients, and in 70.1%, other lung diseases were also present. Bronchiectasis was only diagnosed in 17 patients. In patients >70 yrs the prevalence of COPD with or without emphysema was 80%. In 158 patients, other diagnoses, mainly chronic bronchitis without obstructive lung disease, were present or information was missing.

Results of the dried blood spots in patients with lung diseases

The phenotypes and the corresponding α_1 -Pi concentrations of the 1,060 individuals tested are shown in table 3.

The phenotype was a statistically significant predictor for the α_1 -Pi concentrations ($p<0.0001$). The

Table 3. – α_1 -Pi concentration in relation to phenotype

Phenotype	Subjects n	Mean	Median	Minimum	Maximum
PiM	980	31.67±3.07	>34	21.00	>34
PiMS	36	28.31±5.42	28.70	18.30	>34
PiMZ	39	19.96±4.68	19.20	12.50	31.20
PiM0	1	19.70			
PiS	1	14.50			
PiSZ	3	15.20±2.01	16.10	12.90	16.60

Data expressed in μ M.

Table 4. – Gene frequency by diagnosis

Diagnosis	Subjects n	PiM	PiS	PiZ	Pi0
Asthma	419	0.96897	0.01312	0.01671	0.00119
COPD	390	0.96026	0.01795	0.02179	0
Emphysema	301	0.95847	0.02326	0.01827	0
Bronchiectasis	17	0.97059	0	0.02941	0

COPD: chronic obstructive pulmonary disease.

highest were found in patients with PiM phenotype. They were significantly higher than for PiMS or PiMZ individuals ($p<0.0001$, both comparisons), and these again were significantly higher than for PiSZ ($p<0.05$, both comparisons). No homozygous PiZ and only three PiSZ individuals were found. The percentage of PiMZ for the three most commonly diagnosed lung diseases was 3.9% for COPD, 2.9% for emphysema and 2.9% for asthma, respectively. Patients with acute or chronic bronchitis as the only diagnosis were omitted from the analysis. The gene frequencies stratified for the diagnoses are shown in table 4.

There was no statistically significant difference between the gene frequencies of the alleles associated with α_1 -Pi deficiency S or Z for any given diagnosis. There was also no statistically significant difference between the gene frequencies for reported smoking status.

Discussion

The new test method of quantifying α_1 -Pi concentrations from DBS proved to be very reliable in comparison with conventional methods. In addition, phenotyping samples by a combination of IEF and immunoblotting from DBS showed an accuracy of 100%. In a prospective screening of patients with chronic lung disease, the gene frequency was similar to that found in normal volunteers [14–16]. On the basis of this study the hypothesis that 2–3% of all patients with emphysema are homozygous for PiZ must be refuted in an unselected population seeking primary care for COPD and asthma.

DBS have been used in newborn screening to detect several diseases. They can be easily applied to a large number of individuals, no phlebotomy is required, and storage as well as transportation of samples does not require special containers or precautions. In the classic study by LAURELL and SVEGER [17], 108,000

Swedish infants were screened for α_1 -Pi deficiency using DBS. However, at the time they were still unable to quantify α_1 -Pi concentrations in dried blood and used the height of the transferrin peak as a relative reference. An enzyme-linked immunosorbent assay (ELISA) method similar to the one described in this study was first used in a screening of >39,000 newborns in Belgium [18] and proved to be a reliable method for quantification of α_1 -Pi. The α_1 -Pi protein and deoxyribonucleic acid collected by DBS is stable at room temperature over a period of 1 month [18]. The method is simple and easily automated and therefore well suited for the screening of large numbers of samples. Recently, a Spanish group used eluates of DBS to quantify α_1 -Pi and determine the phenotypes. As in this study, the correlation of α_1 -Pi concentrations between DBS *versus* serum samples was excellent and a total concordance between the phenotyping results was observed [19].

To obtain results on the frequency of severe α_1 -Pi deficiency in a target population of patients with COPD or asthma seeking medical attention in physicians' offices, the current authors tested both for α_1 -Pi concentration and phenotype. This approach gave results for the rate of heterozygotes in the population and thus on the gene frequency of M, S and Z alleles. For the purpose of identifying individuals with severe deficiency only, a two-step approach would be more appropriate, in which it would be sufficient to phenotype individuals below a cut-off value of 20 μ M α_1 -Pi in the serum only. In this case, a high percentage of heterozygous MZ and MS would not be detected. In this study, all samples were both quantitated and phenotyped. A cut-off of 20 μ M would have identified all individuals with PiMO, S and SZ phenotypes. Of the carriers of one deficient allele, 11% of the PiMS and 56% of the PiMZ would have been identified. Thus a cut-off of 20 μ M proved to be a safe method to identify all individuals with severe deficiency.

Several investigations in the 1970s reported a high prevalence of patients with severe α_1 -Pi deficiency in populations of patients with COPD and/or emphysema. In 1969, LIEBERMAN and colleagues [7, 20] investigated the serum trypsin inhibitory capacity (TIC) of 66 individuals admitted to a veterans hospital for pulmonary emphysema. On the basis of TIC alone, they identified 10.6% homozygotes and 15.2% heterozygotes; no phenotyping results were available. To study a possible selection bias by hospitals particularly interested in α_1 -Pi deficiency, investigators studied the phenotypes of 240 patients with COPD and/or emphysema in a referral population and compared them with those obtained by a hospital especially interested in α_1 -Pi deficiency [9]. The percentages of both homozygous (2.5 *versus* 5.4% PiZ individuals) and heterozygous individuals (8.3 *versus* 13.8% PiMZ) in the specialised clinic were significantly higher, confirming a substantial selection bias induced by the specialisation in α_1 -Pi deficiency. Cox *et al.* [8] investigated 163 patients with COPD from the pulmonary service of a large urban hospital and found 4.9% PiZ patients in the whole group and as much as 17.8% PiZ in patients with pulmonary emphysema. In 1986, LIEBERMAN

et al. [7] found 1.9% homozygous PiZ individuals in a population of 965 patients with severe COPD. No details on the population tested were given. There is, however, evidence for a pronounced selection bias. Patients were severely impaired and were referred for bilateral carotid body surgery in a desperate attempt to alleviate dyspnoea.

Based on these reports, the WHO [10] stated that 2–3% of all emphysema patients were homozygous for PiZ and recommended screening for α_1 -Pi deficiency in patients with COPD and emphysema.

One of the aims of this study was to test the validity of the WHO recommendation for screening of populations at risk. The current authors' hypothesis was that by screening patients with lung disease, mainly COPD and emphysema, it would be possible to identify a large number of patients with severe α_1 -Pi deficiency. Using a more conservative estimate of 1–2% homozygous PiZ in the target population in this study would have led to the identification of 10–20 severely deficient individuals in 1,000 screened patients. The fact that no PiZ individuals in 1,060 screened patients were found in this study results in a frequency of homozygous PiZ of <0.1% as opposed to the 1–2% initially expected. Excluding patients with asthma from the analysis, there were 691 patients with COPD and/or pulmonary emphysema resulting in a frequency of homozygotes PiZ of <0.2%.

Even though there are obvious differences in patient selection between the historical studies cited above and the investigation presented here, the lack of severe α_1 -Pi deficient individuals in the population tested was unexpected. In contrast to the other studies, patients referred to a hospital where there is a particular interest in α_1 -Pi deficiency were not studied. Patients diagnosed as having COPD seeking primary care are likely to have less severe disease than patients hospitalised for COPD. In addition, the population of the previous studies was older and had a higher percentage of males. In this study lung function criteria were not used to define COPD. Even though most of the patients in this study had a lung-function test as part of their work-up, there may have been differences in the criteria for diagnosis of COPD. In addition, the use of TIC either alone or with acid starch electrophoresis might have led to an overestimation of the frequency of the Z allele. These factors point toward a significant selection bias in the historical studies investigating the percentage of PiZ individuals in a population of patients with emphysema. This study shows that these results cannot be extrapolated for a population of patients with COPD in a primary care setting and a large-scale screening of this patient group will not yield a 1–2% detection rate of homozygous PiZ. Targeted screenings following the recommendation of the WHO in its current form are not sufficient to increase the detection rate of α_1 -Pi deficiency. Additional selection criteria must be identified and evaluated for a high yield detection programme.

There has been considerable controversy regarding the increased risk of developing emphysema for patients with intermediate α_1 -Pi deficiency. Some studies have found an increased risk of development of COPD in individuals with PiMZ [8, 9, 21], while

others have not [22, 23]. In several population-based studies in Germany using IEF to determine the phenotypes, the percentage of PiMZ was 2.4–3.8%, which was not significantly different from the 3.7% found in this investigation [14–16]. Although this implies that the frequency of heterozygous PiMZ is not increased in a population of patients with COPD, this study was not designed to test this hypothesis. A normal control group in the same geographical area being tested by the same laboratory methods would have been required to further investigate this question.

In conclusion, the dried blood spot method described in this paper is accurate and well suited for the screening of a large population. In contrast to previously published studies, an increased frequency of intermediate α_1 -Pi deficiency in patients with chronic obstructive pulmonary disease was not found. Since no individuals with severe α_1 -Pi deficiency were found, the percentage of patients with PiZ in an unselected population of patients with asthma or chronic obstructive pulmonary disease was <0.1%.

Acknowledgements. The authors would like thank the following participating physicians in Germany and their dedicated staff for their valuable contribution to this study: G. Wichtmann (pulmonary specialist), Recklinghausen; P. Gussmann (general practitioner), Gelsenkirchen; G. Hammelmann (pulmonary specialist), Essen; A. Marx (general practitioner), Gelsenkirchen; M. Emmerich (general practitioner), Gelsenkirchen; W. Marx (general practitioner), Oberhausen; and J. Roghmann (general practitioner), Bottrop.

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