Eur Respir J 2000; 15: 1111–1115
Printed in UK – all rights reserved

European Respiratory Journal
ISSN 0903-1936

Simple method for α_1-antitrypsin deficiency screening by use of dried blood spot specimens

X. Costa*, R. Jardi*, F. Rodriguez*, M. Miravitlles**, M. Cotrina*, C. Gonzalez*, C. Pascual*, R. Vidal**

Simple method for α_1-antitrypsin deficiency screening by use of dried blood spot specimens. X. Costa, R. Jardi, F. Rodriguez, M. Miravitlles, M. Cotrina, C. Gonzalez, C. Pascual, R.. Vidal. ©ERS Journals Ltd 2000.

ABSTRACT: The use of dried blood spot (DBS) specimens in quantitative α_1-antitrypsin (α_1-AT) detection or genetic analysis is limited because protein levels in the samples are low and they contain components that can interfere with polymerase chain reaction amplification. A methodological adaptation was developed to overcome these drawbacks which is discussed here.

The study population consisted of 200 healthy volunteers and 300 patients with chronic obstructive pulmonary disease (COPD). DBS specimens were tested for α_1-AT concentration using a modified nephelometric assay and phenotyped with an isoelectric focusing method. Genetic diagnosis was established by deoxyribonucleic acid sequencing using a simple purification procedure to remove contaminants.

The nephelometric method showed a detection limit of 0.284 mg·dL^{-1}, corresponding to a serum concentration of 13 mg·dL^{-1}. The correlation coefficient between α_1-AT concentrations in DBS *versus* serum samples was R^2=0.8674 ($p<0.0001$). All 200 healthy individuals had DBS α_1-AT concentrations >1.9 mg·dL^{-1}, corresponding to 114 mg·dL^{-1} in serum samples. One hundred and twenty-five COPD patients (42%) showed α_1-AT values <1.8 mg·dL^{-1}. Twenty patients with the PIZ phenotype had α_1-AT values lower than 0.64 mg·dL^{-1}. On the basis of genotyping, one COPD patient was classified as heterozygous (PIMM$_{heerlen}$).

Selective elution of contaminants resulted in optimal $\alpha_1$1-antitrypsin genotyping. Because of its sensitivity and excellent correlation with the standard method, the dried blood spot quantitative assay is a reliable tool for routine measurement of α_1-antitrypsin.

Eur Respir J 2000; 15: 1111–1115.

Depts of *Biochemistry and **Pneumology, Hospital Universitario Vall d'Hebron, Barcelona, Spain.

Correspondence: R. Jardi, Servicio de Bioquimica, Hospital General Vall d'Hebron, Paseo Vall d'Hebron 119-129, Barcelona 08035, Spain. Fax: 34 932746831.

Keywords: α_1-Antitrypsin deficiency
α-antitrypsin quantification
α-antitrypsin screening
dried blood spot
genotype
phenotype

Received: November 3 1999
Accepted after revision February 9 2000

This project was supported, in part, by a grant from the Fundació Marató TV3 and by the "Maria Ravà" grant from the Fundació Catalana de Pneumologia (FUCAP).

α_1-antitrypsin (α_1-AT) deficiency is a hereditary autosomal codominant disorder resulting from mutations in the α_1-AT gene. It is characterized by reduced serum concentrations of α_1-AT and is associated with a high risk for the development of early-onset pulmonary emphysema, occasionally with liver damage [1].

α_1-AT is a highly polymorphic protein; >70 genetically determined variants (called proteinase inhibitor (PI) types) have been identified. The PIM variant and its serum subtypes are the most common of the normal alleles. PIZ is the most important allele associated with reduced concentrations of plasma α_1-AT and a significant risk of developing disease [2, 3].

The current approach to laboratory diagnosis of α_1-AT deficiency uses a combination of serum α_1-AT measurement and identification of the α_1-AT phenotype by the isoelectric focusing (IEF) pattern at pH 4.2–4.9 [4]. More recently, methods such as the polymerase chain reaction (PCR) and simple deoxyribonucleic acid (DNA)-based methods for assigning the most common deficient alleles have been incorporated into these studies [5, 6]. Although diagnosis of deficiency is relatively simple, population studies indicate that α_1-AT deficiency is underdiagnosed and that prolonged delays in diagnosis are common [7, 8]. Recent recommendations of the World Health Organization advocate screening programmes using a quantitative test, especially among patients with chronic obstructive pulmonary disease (COPD) and adults and adolescents with asthma. Patients with abnormal results on screening should undergo PI typing [9].

Dried blood spot (DBS) specimens have been employed for genetic screening and diagnosis of several diseases [10]. However, their use poses considerable technical obstacles in the study of α_1-AT deficiency. α_1-AT quantification is affected by haemoglobin, and in genetic analysis the low purity of DBS DNA and the natural PCR inhibitors that are present can cause interference [10, 11]. In the present study, a simple specific immune nephelometric method for the quantitative determination of α_1-AT in DBS samples was evaluated. In addition, to facilitate α_1-AT genotyping in these samples, a simple purification procedure was applied to remove contaminants before the PCR. The results obtained in DBS specimens were compared to those from serum or fresh blood samples in blinded experiments. The complete protocol employed for α_1-AT deficiency screening is described.

Patients and methods

Patients

From June 1998 to June 1999, a total of 300 COPD patients, 20 of whom were known to be α_1-AT deficient (phenotype PIZ), were recruited for the study, together with a second group of 200 healthy volunteers. DBS and serum specimens were obtained at the same time for each subject.

Quantitative determination of α_1-antitrypsin levels

To prepare the DBS, ~30 µL of capillary blood was applied to individual 3-mm paper discs (No. 903; Schleicher & Schuell, Dassel, Germany). The discs (three per patient) were left to dry at room temperature (22°C) prior to storage in plastic bags at -20°C until use.

For the dried blood quantitative assay, one disc per patient was eluted with 200 µmL 10 mM phosphate-buffered saline (pH 7.4) overnight at 4°C. Subsequently, each sample was centrifuged at 1,000×*g* for 1 min and α_1-AT levels were determined in the eluate using a rate immune nephelometric method (Immage Immuno Chemistry System, Beckmann, Fullerton, CA, USA). When working with serum samples, the immune nephelometer used automatically dilutes samples 1:36 to achieve optimum antigen/antibody equilibrium in the assay. However, DBS samples contain a much smaller volume of blood and α_1-AT absolute values are much lower, making dilution unnecessary. Thus the manufacturer's protocol was adapted to work without dilution before determining α_1-AT levels in the DBS samples. Haemoglobin interference, measured by adding known concentrations of haemoglobin to DBS eluates, was not observed at concentrations of <200 mg·dL^{-1}.

The normal range for α_1-AT in serum samples was 114–170 mg·dL^{-1}.

α_1-antitrypsin phenotyping

One DBS disc from each patient was eluted with 30 µL specimen diluent (60 µM cysteine, 1 M glycine, pH 7.4) overnight at 4°C. Screening for α_1-AT variants was carried out in 5 µL of eluate by use of an IEF technique using carrier ampholytes on flat bed polyacrylamide gels in a pH gradient of 4.2–4.9, as described previously [12]. After silver staining according to the manufacturer's recommendations (Protein Silver Staining Kit; Pharmacia, Uppsala, Sweden), the α_1-AT bands were examined for phenotype. In each assay, controls consisting of DBS samples corresponding to the PIM, PIMS and PIMZ phenotypes were included. To obtain good reproducibility and optimum clarity of the major IEF band migrations, it is important to stop the silver reaction at the point in time when the weakest control band (PIZ variant) starts becoming visible.

α_1-AT phenotyping in serum samples was carried out as previously reported [12].

α_1-antitrypsin genotyping by polymerase chain reaction and direct deoxyribonucleic acid sequencing

For the molecular analysis, one 3-mm disc was cut into four parts and each part was placed in a separate 1.5 mL plastic tube. The forceps used for this procedure were sterilized each time by dipping in ethanol and flaming to avoid cross contamination of specimens. To eliminate PCR-inhibiting substances, the disc fragments were washed twice with 1 mL of water by inverting the tube several times, according to a method described by MAKOWSKI *et al.* [13]. After this simple wash, each disc fragment was transferred into a separate PCR reaction tube containing 25 µL water and heated in a PCR thermal cycler for 10 min at 99°C. Subsequently, the four exons of the α_1-AT gene were amplified by PCR (45 thermal cycles) and directly sequenced. The reaction conditions and the PCR and sequencing procedures have been described previously [14].

Stability of dried blood spot specimens

The DBS samples were stored at room temperature, 4°C and -20°C and then processed for the three assays at intervals of 1, 2, 3 and 4 weeks.

Statistical analysis

Values corresponding to those predicted and absolute errors were calculated as mean and 95% confidence interval. The correlation between serum and DBS α_1-AT concentrations obtained with the rate immune nephelometric method was studied with linear regression analysis. The statistical package for social sciences (SPSS, Chicago, IL, USA) statistics program, version 7.5, was used for statistical analyses.

Results

Experiments designed to assess the accuracy of the DBS quantitative α_1-AT assay were conducted with specimens from two patients, one with the PIM (normal α_1-AT level) and the other with the PIMZ (low α_1-AT level) phenotype. The intra- and interassay variation coefficients were 4.5 and 9.8% (for PIM) and 4.1 and 9.3% (for PIMZ).

DBS specimens and serum samples from 143 patients with different α_1-AT phenotypes were analysed to obtain α_1-AT concentrations spanning the useful range of the nephelometric assay. In order to calculate the best regression line, the square root of the DBS α_1-AT concentration ($x^{0.5}$) was used. The estimated regression line was $y = -50.943 + 118.59x^{0.5}$ (fig. 1). The correlation coefficient between α_1-AT levels using DBS *versus* serum samples was $R^2 = 0.8674$ ($p<0.0001$). The mean prediction error and mean absolute error were 2.4% (95% confidence interval (CI) 0.45–5.25) and 10.98% (95% CI 9.46–12.5), respectively. The detection range of the DBS nephelometric assay was 0.284–2.84 mg·dL^{-1}, corresponding to 13–160 mg·dL^{-1} α_1-AT in serum according to the regression curve. Samples with concentrations >2.84 mg·dL^{-1} were diluted and reprocessed.

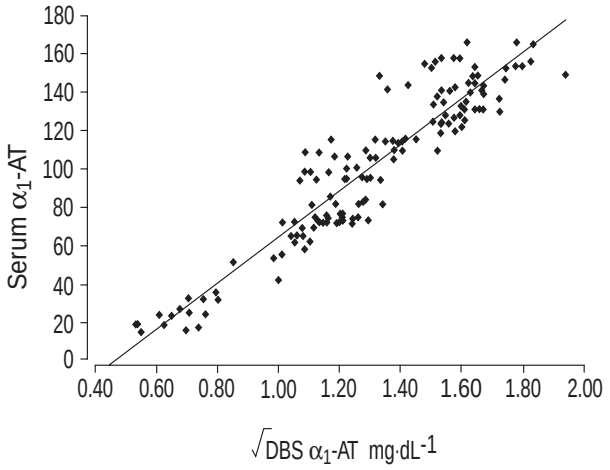

Fig. 1. – Quantitative determinartion of α_1-antitrypsin (α_1-AT) levels by the immune nephelometric method. Correlation between serum and dried blood spot (DBS) α_1-AT levels in specimens from 143 patients. $y=118{\cdot}59x^{0.5} - 50.943$, $R^2=0.8674$.

All the 200 healthy individuals had DBS α_1-AT levels of 1.9 mg·dL^{-1}. One hundred and seventy-nine (89.5%) cases had the phenotype PIM, 20 (10%) PIMS and one (0.5%) PIMZ. Among the 300 patients studied, 125 (42%) had DBS α_1-AT concentrations of <1.8 mg·dL^{-1}. Three (2%) had the phenotype PIM, 28 (27%) PIMS, 71 (56%) PIMZ, one (1%) PIS, two (2%) PISZ and 20 (16%) PIZ. Total concordance was observed between the phenotype results obtained using the DBS samples and those obtained using the serum samples. The relationship between serum α_1-AT concentrations, DBS α_1-AT concentrations and α_1-AT phenotypes is shown in figure 2.

α_1-AT genotype determination was carried out using DNA from DBS and fresh blood samples from four patients with different phenotypes (PIM, PIMZ, PIMS and PIZ). The PCR products and the sequencing peaks obtained from DBSs were as sharp as those of fresh blood, and identical genotypes were obtained in all cases. One

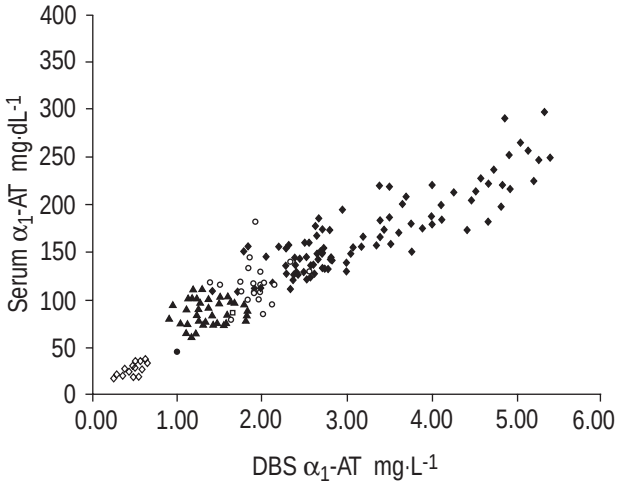

Fig. 2. – α_1-antitrypsin (α_1-AT) levels in serum and dried blood spot (DBS) specimens obtained from patients with chronic obstructive pulmonary disease, relationship with the phenotype (◆: proteinase inhibitor (PI) M; ○: PIMS; ▲: PIMZ; □: PIS; ●: PISZ; ◇: PIZ).

patient with COPD showed DBS α_1-AT levels of 1.4–1.6 mg·dL^{-1} at various intervals. In the phenotype study he was classified as phenotype PIM. However, since α_1-AT concentration and phenotype were not concordant (the α_1-AT DBS level of 1.6 mg·dL^{-1}, corresponding to 70 mg·dL^{-1} in serum, is low for the PIM phenotype), DNA analysis was performed. All the α_1-AT exons (II–IV) were amplified and the PCR products sequenced, as previously described [12]. Analysis of the DNA sequence showed a change from CCC to TCC at codon 369 in exon V. This substitution is characteristic of the heerlen PIM (PIM$_{heerlen}$) α_1-AT variant [15]. On the basis of genotyping, the patient was classified as heterozygous PIMM$_{heerlen}$. A fragment of the α_1-AT exon V sequence around residue 369, obtained from DBS DNA, is shown in figure 3.

Storage of the DBS samples showed no *in vitro* destruction at room temperature over a period of 1 week. Samples stored at 4°C and -20°C were assayed weekly for 4 weeks and no significant decrease in α_1-AT concentration, degradation of phenotyping IEF bands or alterations were observed.

Discussion

The automated immune nephelometric quantitative analysis of α_1-AT is a simple and sensitive method with excellent correlation between DBS specimens and serum sample results, making it suitable for routine screening of hereditary α_1-AT deficiency. Quantitative determination of serum α_1-AT levels is an important first step in the diagnosis of α_1-AT deficiency. This is difficult in DBS samples, in which protein concentration is low and certain blood components can interfere with the analysis [10]. Several assays have been developed for studying α_1-AT levels in DBSs. The semiquantitative methods used for this purpose present limitations for the interpretation of results; and the quantitative methods (*e.g.* radial immunodiffusion and rocket immunoelectrophoresis) are complex and difficult to use with large series of samples [10, 11, 16]. The quantitative immune nephelometric assay developed in this study is less time-consuming and can be automated for large numbers of samples. Moreover, no interference due to haemoglobin contamination was found. Through the regression line, it was possible to estimate α_1-AT concentrations in serum from DBS concentrations, allowing use of the serum reference range as the normal range for both methods.

In the DBS screening protocol proposed, deficiency is evaluated by combining the results of α_1-AT quantification and α_1-AT phenotyping. In cases in which there is discordance between α_1-AT concentration and phenotype, diagnosis of hereditary α_1-AT deficiency is established by α_1-AT genotyping. With the simplified procedure used to study the α_1-AT genotype, DNA extraction is not required and the use of organic solvents is avoided. In addition, the protocol overcomes the problems of PCR-inhibiting substances in DBS DNA samples by selective elution of contaminants before the PCR assay.

A sequencing method was used to study DNA polymorphism instead of a simpler allele-specific PCR because the more common deficient variants, PIS and PIZ, are always well-identified by the IEF phenotyping method and the rare α_1-AT deficient variants that are not recognized by

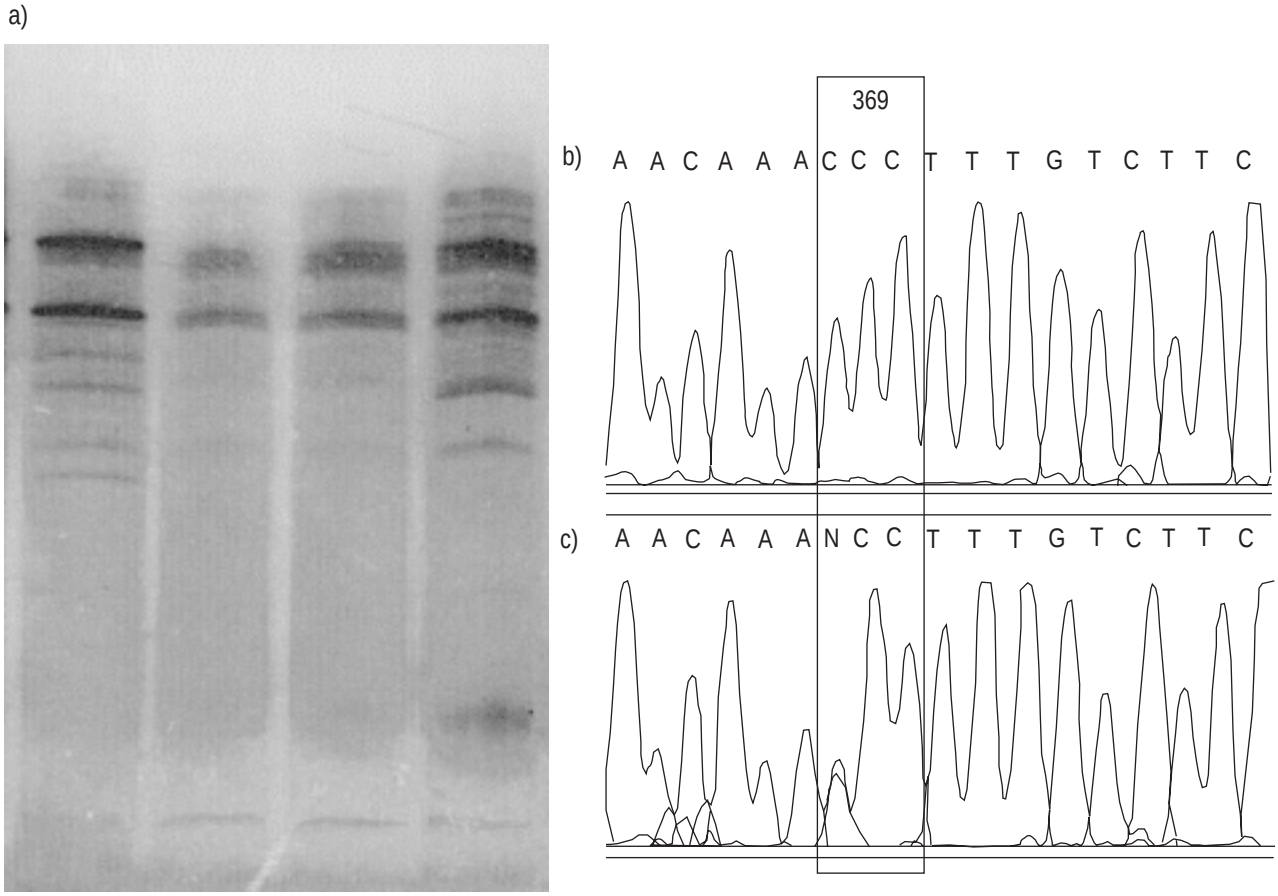

Fig. 3. – Characterization of heerlen proteinase inhibitor (PI)M (PIM$_{heerlen}$) variant. a) Isoelectric focusing gel of α_1-AT phenotypes (pH range 4.2–4.9) obtained from dried blood spot (DBS) specimens. The anode is at the top. The normal isoprotein major band migration areas 4, 6 and 8 for α_1-AT are shown (m4, m6 and m8). Lane: 1: PIMZ; lane 2: PIMM$_{heerlen}$, lane 3: PIM; lane 4: PIMS. The PIMM$_{heerlen}$ and PIM variants show a similar isoelectric point and therefore the same position in m4 and m6. b, c) Direct sequencing of the α_1-AT gene from deoxyribonucleic acid obtained from DBS specimens: b) fragments of exon V around position 369 from an individual without the mutation; and c) the corresponding fragment of exon V from a heterozygous PIMM$_{heerlen}$ patient. PIM$_{heerlen}$ in exon V differs from the normal M variant by mutation of CCC to TCC. A: adenine; C: cytosine; G: guanine; T: thymine; N:C/T heterozygous.

IEF must always be identified by complete study of the four α_1-AT exons. The value of α_1-AT genotyping by sequencing was demonstrated in the present study by identification of the rare deficient PIM$_{heerlen}$ variant, characterized by an allelic background, M1(valine^{213}), and a mutation of CCC to TCC in exon V of the α_1-AT gene [15]. The patient presented low levels of α_1-AT and had been phenotyped erroneously as PIM because the PIMM-$_{heerlen}$ and PIM variants show a similar isoelectric point and are difficult to differentiate using IEF methodology. The present genotyping study characterized this patient as heterozygous PIMM$_{heerlen}$.

The allelic frequency of PIZ, the variant most commonly associated with the deficient state, is 0.01–0.02 in the Mediterranean area and 0.02–0.03 in Northern Europe [17]. In a recent study, it was found that the allelic frequency of PIZ in North-East Spain was 0.15, one of the highest in Spain [18]. The expected frequency of PIZ individuals in this area is therefore 225 per million. In general, the number of individuals definitely diagnosed with deficiency is far lower than expected, suggesting that many individuals with the condition are either undiagnosed or misdiagnosed [7–9]. A recent study has recommended that neonatal screening programmes should be undertaken in developed countries with a Caucasian population, and that all patients with COPD and adults and adolescents with asthma be screened once for α_1-AT deficiency using a quantitative test, to identify susceptible individuals before onset of disease. Those with abnormal results on screening should then undergo PI typing [7]. The test described here permits early diagnosis and genetic counselling, with the opportunity for lifestyle adaptations and potentially life-saving therapy. In the general population, it has been demonstrated that patients diagnosed early through family studies have a better prognosis than those diagnosed after the onset of symptoms [19].

The results of the present study indicate that dried blood spot specimens provide a reliable alternative to plasma for α_1-antitrypsin screening and a valuable source of deoxiribonucleic acid for the detection of genetic diseases. The dried blood spot method does not require elaborate specimen collection equipment and the samples obtained are easy to store and transport, thereby providing an excellent option for studying the frequency of deficient alleles in

large populations. The α_1-antitrypsin quantification procedure described is simple, sensitive and correlates perfectly with the standard technique. Because the protocol is less time-consuming, results from the three tests can be obtained within 2 days after receiving the specimen.

Acknowledgements. The authors thank M. Gimferrer and I. Gil for their collaboration in the realization of this work.

References

1. Carrel RW, Lomas DA, Sidhar S, Foreman R. α_1-Antitrypsin deficiency. A conformational disease. *Chest* 1996; 110: 243S–247S.
2. Mastrangeli A, Crystal RG. Alpha 1-antitrypsin. An introduction. *In*: Crystal RG, ed. Alpha 1-Antitrypsin Deficiency. Biology. Pathogenesis. Clinical Manifestations. Therapy. New York, Marcel Dekker, Inc. 1996; pp. 3–18.
3. Eriksson S. A 20-year perspective on α_1-antitrypsin deficiency. *Chest* 1996; 110: 237–242.
4. Blank CA, Brantly M. Clinical features and molecular characteristics of α_1-Antitrypsin deficiency. *Ann Allergy* 1994; 72: 105–111.
5. Brantly M. Alpha 1-antitrypsin genotypes and phenotypes. *In*: Crystal RG, ed. Alpha 1-Antitrypsin Deficiency. Biology. Pathogenesis. Clinical Manifestations. Therapy. New York, Marcel Dekker, Inc. 1996; pp. 45–59.
6. Faber JP, Poller W, Weidinger S, *et al*. Identification and DNA sequence analysis of 15 new α_1-antitrypsin variants, including two PI*Q0 alleles and one deficient PI*M allele. *Am J Hum Genet* 1994; 55: 1113–1121.
7. McElvaney NG, Stoller JK, Buist AS, *et al*. Baseline characteristics of enrolees in the National Hearth, Lung and Blood Institute Registry of alpha-1-antitrypsin deficiency. *Chest* 1997; 111: 394–403.
8. Miravitlles M, Vidal R, Barros-Tizón JC, *et al*. Usefulness of a national Registry of alpha-1-antitrypsin deficiency. The Spanish experience. *Respir Med* 1998; 92: 1181–1187.
9. Anonymous. Alpha-1-antitrypsin deficiency: memorandum from a WHO meeting. *Bull World Health Organ* 1997; 75: 397–415.
10. Caggana M, Conroy JM, Pass KA. Rapid, efficient method for multiplex amplification from filter paper. *Hum Mutat* 1997; 11: 404–409.
11. Schoos R, Dodinval Versie J, Verloes A, Lambotte C, Koulischer L. Enzyme immunoassay screening of alpha-1-antitrypsin in dried blood spots from 39,289 newborns. *Clin Chem* 1991; 37: 821–825.
12. Jardi R, Rodriguez F, Casas F, *et al*. Caracterización molecular de dos variantes deficitarias de la Alfa 1-antitripsina. PI Mpalermo y PIPlovel. *Med Clin (Barc)* 1997; 109: 463–466.
13. Makowski GS, Davis EL, Aslanzadeh A, Hopfer SM. Enhanced direct amplification of Guthrie card DNA following selective elution of PCR inhibitors. *Nucleic Acids Res* 1995; 23: 3788–3789.
14. Jardi R, Rodriguez F, Miravitlles M, *et al*. Identification and molecular characterisation of the new alpha-1-antitrypsin deficient allele PIYBarcelona (Asp256-Val, Pro-391-His). Mutation in brief No. 174. Hum Mutat 1998; http:journals.wiley.com/1059-7794/pdf/mutation/174.pdf.
15. Hofker M, Nukiwa T, Paassen H, *et al*. A Pro-Leu substitution in codon 369 of the alpha-1-antitrypsin deficiency variant PI Mheerlen. *Hum Genet* 1989; 81: 264–268.
16. Jeppsson JO, Sveger T. Typing of genetic variants of alpha-1-antitrypsin from dried blood. *Scand J Clin Lab Invest* 1984; 44: 413–415.
17. Hutchinson DCS. Alpha-1-antitrypsin deficiency in Europe: geographical distribution of PI types S and Z. *Respir Med* 1998; 92: 367–377.
18. Vidal R, Miravitlles M, Jardi R, *et al*. Estudio de la frecuencia de los diferentes fenotipos de la alfa-1-antitripsina en una población de Barcelona. *Med Clin (Barc)* 1996; 107: 211–214.
19. Seersholm N, Kok-Jensen A, Dirksen A. Survival of patients with severe alpha 1- antitrypsin deficiency with special reference to index cases. *Thorax* 1994; 94: 659–698.