

## Automated Determination of Serum $\alpha$ 1-Antitrypsin by Antitryptic Activity Measurement

Denis Roche,<sup>1\*</sup> Alexandra Mesner,<sup>1</sup> Malik Al Nakib,<sup>1</sup> Frederic Leonard,<sup>1</sup> and Philippe Beaune<sup>1</sup>

**BACKGROUND:**  $\alpha$ 1-Antitrypsin (A1AT) deficiency is currently detectable by protein immunoassay, phenotyping, and genotyping of the S and Z mutations, but no fully automated method for standard biochemical analyzers is yet available. Here, we present a method that measures the antitryptic activity in serum. This method is rapid, automated, and allows the easy evaluation of a large cohort of patients.

**METHODS:** Our automated assay involves determining serum antitryptic capacity on the Olympus AU 400 autoanalyzer by using trypsin and succinylated gelatin as substrate in the presence of trinitrobenzene sulfonic acid. The results are expressed as a percentage of inhibition of the reaction of trypsin with succinylated gelatin. After we performed analytical validation studies and reference-interval determination based on serum samples from 120 healthy persons, we tested the assay on deidentified samples from 120 patients with various pathologies (primarily pulmonary) of unexplained origin and normal A1AT concentrations and phenotypes.

**RESULTS:** The analysis rate was up to 120 samples per hour. Intraassay CVs ranged from 3.1%–16.2%, and interassay CV was 7.5%. The reference population showed mean (SD) 58.4 (6.7)% inhibition. The detection limit was 9.5% inhibition. The 120 studied patients displayed significantly lower mean activity than 120 healthy individuals ( $P < 0.0001$ ).

**CONCLUSION:** This assay is stable, reliable, and easily automated by use of open-system analyzers, allowing for the rapid evaluation of patients. After further validation on a larger randomized cohort, this new approach should function as a useful method to explore A1AT deficiency, especially in large-scale studies.

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$\alpha$ 1-Antitrypsin (A1AT)<sup>2</sup> is the most abundant protein, on the basis of molar concentration, in the serpin fam-

ily (1). This 52-kDa glycoprotein is primarily synthesized in the liver and found in the plasma, where it performs 90% of the antiprotease activity, primarily as an inhibitor of leukocyte elastase. A1AT deficiency, which is most often of genetic origin but frequently goes undiagnosed or misdiagnosed, is associated with various pathologies of the lung, liver, and, more rarely, skin (2–4). As many as 100 genetic variants have been described (5–7). The wild-type allele is the M allele; the main variants associated with deficiencies are the S allele (E288V; allelic frequency 5–150 per 1000 in Europe), and the Z allele (E366K; 0–30 per 1000 in Europe). The latter mutation leads to polymerization of the protein in the hepatocyte (8), resulting in a deficiency of A1AT secretion, accumulation in the hepatocyte (potentially leading to cytolysis in homozygous individuals) and an imbalance between protease and antiprotease activities in the lung. Phenotyping is performed by isoelectric focusing to identify mutations, and genotyping is done by PCR for the S and Z mutations. Some patients live with this deficiency without exhibiting any pathological symptoms, suggesting that their antiprotease activity is efficient enough to maintain the protease/antiprotease balance. Conversely, with some pathologies that are linked to elastase but are of undefined etiology, affected individuals display A1AT concentrations within reference intervals and apparently lack a mutation. In these cases, there may be a decrease in the antiprotease activity without a change in the quantity of the protein. An investigation of this hypothesis requires the study of large cohorts of patients and, therefore, the availability of a rapid and low-cost assay. Assays for the activity of proteolytic enzymes were developed in the 1960s; the inhibition of proteolytic enzyme activity was then used to measure the antiprotease activity of serpins, particularly the most abundant of these, A1AT and  $\alpha$ 2-macroglobulin (9). The principle of the reaction has not changed since its development, and the only modifications have been in the choice of the substrate: synthetic (10), chromo-

<sup>1</sup> AP-HP, Hôpital Européen Georges Pompidou, Service de Biochimie, 75015 Paris, France

\* Address correspondence to the author at: APHP, Hôpital Européen Georges Pompidou, 20-40 rue Leblanc, Paris Cedex 15, F 75908, France. Fax 331-156093393; e-mail denis.roche@egp.aphp.fr.

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<sup>2</sup> Nonstandard abbreviations: A1AT,  $\alpha$ 1-antitrypsin; TNBSA, 2,4,6-trinitrobenzenesulfonic acid; A, absorbance; MB, enzymatic reaction mode; BAL, bronchoalveolar lavage; AU, absorbance units; LOD, limit of detection.

fluorogenic (such as *n*- $\alpha$ -benzoyl-DL-arginine-p-nitroanilid), or natural (such as elastin or gelatin) (11). These techniques have been partially automated, but a manual predilution of the samples is still required (12, 13); these methods have also been adapted to 96-well microplates for a limited series of studies (14). A turbidimetric method (15), based on a dose-dependent rate of trypsin-mediated dissociation of aggregates of fibrin-coated, microscopic beads has been proposed recently. Because the latter method requires sample cells to be continuously stirred at 1000 rpm, it cannot be adapted for high throughput in standard automated biochemistry analyzers. The aim of this study was to develop an automated assay of serum antitryptic capacity to be performed on the Olympus AU400 auto-analyzer. Our method was based on an existing method that uses trypsin and succinylated gelatin in the presence of trinitrobenzene sulfonic acid (16, 17). This method was modified to conform to specifications of the most frequently used autoanalyzers, i.e., the addition of no more than 2 reagents to the sample.

## Materials and Methods

### CHEMICALS

Sodium hydroxyde and hydrochloric acid were purchased from VWR International. Succinic anhydride and sodium tetraborate were purchased from Sigma. We diluted 2,4,6 trinitrobenzene sulfonic acid (TNBSA) 5% (Sigma P2297) before use in borate buffer 0.04 mol/L (sodium tetraborate + hydrochloric acid sufficient for pH 8.5) to a final concentration of  $3 \times 10^{-4}$  mol/L; 0.5 g/L porcine trypsin, minimum 250 USP units/mg in 0.2% EDTA (Eurobio), was diluted to 0.025 g/L in borate buffer (pH 8.5) and stored at  $-20^\circ\text{C}$ . Gelatin 2% solution (Sigma G 1393) was succinylated using a standard procedure described by Rao and coworkers (11). Briefly, 20 mL of gelatin 2% was diluted with 10 mL borate buffer, followed by addition of 500 mg of succinic anhydride in very small increments to the solution while it was stirred with a magnetic stirrer. The pH of the reaction was maintained between 8.0 and 8.5 by the addition of 1 mol/L NaOH. Excess succinic acid was removed by extensive dialysis (12.4-kDa molecular weight cutoff) overnight against borate buffer. Total protein concentration was measured by quantitative colorimetry (562 nm) by using the BCA protein assay kit (Pierce Chemical) (18) and adjusted to 0.75 g/L with borate buffer. The succinylated gelatin was stored at  $-20^\circ\text{C}$ .

### INSTRUMENT AND METHODS

Antitryptic capacity was measured on an Olympus AU 400 automated analyzer, although other automated biochemistry analyzers theoretically could also be used

with this method. The total proteolytic capacity of the trypsin solution toward the succinylated gelatin preparation was first established by measuring the free amine release in 3 successive samples in the absence of serum [absorbance( $A_{100\%}$ )]. Free-amine concentrations were then measured in the presence of serum ( $A_{se}$ ). Blanks were obtained by measuring free amine in the absence of succinylated gelatin ( $A_{fa}$ ), and the inhibition of the proteolytic capacity was expressed as the actual reduction in free-amine release in the presence of serum, taking into account the free-amine concentration of the serum.

The Olympus AU400 analyzer was programmed according to the manufacturer's recommendations. Briefly, 3 user-defined tests were programmed: (a) for the measurement of the antitryptic activity of serum,  $A_{se}$  (sample volume = 2, R1 = 200, R2 = 200; R1 was succinylated gelatin 0.75 g/L and R2 was trypsin 0.025 g/L + TNBSA  $3.10^{-4}$  mol/L, prepared daily); (b) for the detection of free primary amines,  $A_{fa}$  (sample volume = 2, R1 = 200, R2 = 200; R1 was borate buffer and R2 was trypsin 0.025 g/L + TNBSA  $3.10^{-4}$  mol/L); and (c) for the measurement of the  $A_{100\%}$  (calibration type = AA, concentration = 100). The absorbance was measured at 450 nm, with the following instrument parameter settings: the method "FIXED 1" with "Point 1 first" = 12, "last" = 27, and "calibration type" = enzymatic reaction mode (MB). Furthermore, the interitem calculation parameters ( $A = 1$ ;  $B = A_{se}$ ;  $C = A_{fa}$ , and  $D = A_{100\%}$ ) and the calculation formula: activity(%) =  $1 - [(A_{se} - A_{fa}/A_{100\%}) \times 100]$  were specified in the "Calculated Tests".

### ANALYTICAL VALIDATION

The A1AT concentration was measured with an immunonephelometric assay on the BN2 analyzer (Dade-Behring). Concentrations of A1AT are presented as micromoles per liter and grams per liter (reference values 19.2–38.4  $\mu\text{mol/L}$  and 1.00–2.00 g/L). Data are expressed as mean (SD). To validate the replacement of elastase by trypsin (which is less expensive and easily available), we followed the evolution of the absorbance at 450 nm of the enzyme/TNBSA mixture in borate buffer at the concentrations used in the test, for 1 hour at  $37^\circ\text{C}$ . This test was carried out 10 times.

All tests were carried out on serum. A comparison between serum and plasma on 20 samples did not show any substantive difference ( $r^2 = 0.9882$ ). The within-run imprecision (CV) was established on 3 anonymized samples of serum at high (38.4  $\mu\text{mol/L}$  and 2.00 g/L), medium (26.9  $\mu\text{mol/L}$  and 1.40 g/L) and low (7.7  $\mu\text{mol/L}$  and 0.40 g/L) concentrations of A1AT. Each sample was assayed 20 times in the same run. The between-run imprecision was established on the medium concentration sample (20 days). Linearity was estab-

lished by triplicate analysis of 10 dilutions of 1 patient sample with a very high antiprotease activity (expected values between 3.24% and 81% inhibition). In this sample, the A1AT concentration was found to be 28.6 (7.7)  $\mu$ mol/L and 1.49 (0.40) g/L. To estimate reference values we used deidentified serum from 120 healthy individuals [60 male, 60 female, age 47 (17) years] with a PiMM phenotype who were free of pulmonary or hepatic pathology and had normal electrolyte and hepatic marker profiles (Healthy PiM). We also tested the application of this technique to bronchoalveolar lavage (BAL) fluid on 70 anonymized BAL fluids from patients in a medical intensive care unit, with specimen volumes varying from 2 to 50  $\mu$ L.

Some mutations in the A1AT gene, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (*SERPINA1*), particularly those resulting in the PiZ phenotype, cause a polymerization of the protein. We investigated the influence of polymerization on antitryptic capacity measured by our technique by treating 4 sera of normal A1AT concentration and activity and PiM phenotype at 60 °C for 3 hours, following the technique of polymerization suggested by Lomas and coworkers (19). First we checked the effectiveness of polymerization by isoelectric focusing on a polyacrylamide gel (Ampholine PAG plate, pH 4.0–5.0; GE Healthcare) and then we measured the antitryptic capacity of the 4 samples.

#### PATIENTS STUDIED

Measurement of activity with our technique was initially performed on 156 deidentified samples [89 male, age 38 (23) years; 67 female, age 45 (22) years] submitted to our center for a laboratory diagnosis of A1AT deficiency. At least 65% of these individuals were smokers. These samples were divided into 3 classes: (i) 11 patients with characterized A1AT deficiency [4.8 (1.3)  $\mu$ mol/L and 0.25 (0.07) g/L] and phenotype PiZZ (Patients PiZZ); (ii) 26 deficient PiMZ patients with documented low concentrations of A1AT [14.8 (3.7)  $\mu$ mol/L and 0.77 (0.19) g/L], suffering from pulmonary pathologies (Patients PiMZ); (iii) 120 PiM patients (Patients PiM) affected with various pathologies of unexplained origin but all compatible with low A1AT activities in spite of normal concentrations of the protein [26.5 (6.7)  $\mu$ mol/L and 1.38 (0.35) g/L], and suffering from various pathologies potentially related to A1AT (chronic obstructive pulmonary disease, n = 68; hepatitis, panniculitis, vasculitis, pancreatitis, glomerulonephritis, n = 52). We used the Kruskal–Wallis 1-way ANOVA followed by the Dunn multiple post hoc test to compare the measured antitryptic capacity of the patient groups to those obtained from the 120 healthy reference individuals previously mentioned (Healthy PiM).

## Results

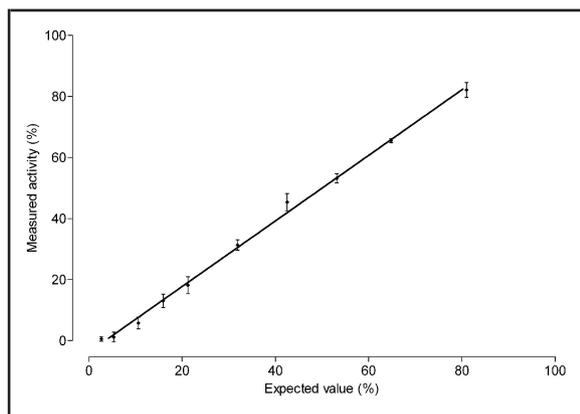
#### ASSAY EVALUATION

We were able to analyze up to 120 samples per hour with the assay as implemented on the Olympus chemistry analyzer. The variation in absorbance measured during the assay ranged from about 0.12 absorbance units (AU) in samples with low antitryptic activity to about 0.20 AU in samples with high activity. The antitryptic capacity of biological fluids was expressed as a percentage of inhibition of the reaction of trypsin on gelatin in the absence of A1AT.

*Choice of substrate.* Synthetic substrates (small molecules), sometimes conjugated to polyethylene glycol to increase their size (20), have been suitable for the measurement of tryptic–antitryptic activity on vegetal products (i.e., soybean trypsin). When it comes to assays in human sera, however, substrates of mammalian origin have a closer resemblance to those encountered in vivo. Elastin should therefore be the best substrate for the present purpose, although it has the major drawback of being a fibrous protein similar to collagen, insoluble in water and only useable after partial hydrolysis to yield pseudosolutions. Because of these drawbacks, elastin has no advantage over gelatin, which is hydrolyzed collagen that is also pseudosoluble in water and much easier to use. Therefore, for practical reasons (easy supply and reliable composition) we used gelatin in our assay.

*Choice of enzyme.* The change in absorbance of the enzyme/TNBSA mixture over the course of the reaction is insignificant for trypsin but very significant (more than 0.30 AU) for elastase. This characteristic ruled out the use of elastase in the presence of TNBSA and is the reason why we used trypsin, although it is less specific for the elastin substrate. Moreover, elastase is expensive and can be difficult to obtain. Fortunately, trypsin and elastase have the same active site and both cleave proteins at lysyl or arginyl residues. Thus, they act on gelatin in the same way, the only difference being that the affinity of elastase for A1AT is a thousand times higher than that of trypsin (21). But this difference, which corresponds to only a fraction of a second, is insignificant in the in vitro reaction in which there is no competition and contact between the enzyme and substrate extends for minutes.

The technical conditions were optimized for the AU400 autoanalyzer, particularly the 2- $\mu$ L minimum sample size, the 8-min maximum incubation time of the reaction, and the reaction temperature of 37 °C. We studied the influence of the reaction temperature (between 25 °C and 47.5 °C) on a medium-activity sample, with a 1.65% CV. We also evaluated the stability of



**Fig. 1.** Linearity of the antitryptic activity assay ( $y = 1.1075x - 3.7814$ ;  $r^2 = 0.9971$ ).

this same sample during 6 days of storage at room temperature and observed a decrease in activity of 8.58%. This small decrease suggests that samples stored at +4 °C or -20 °C for 1 week will likely show no decrease in activity.

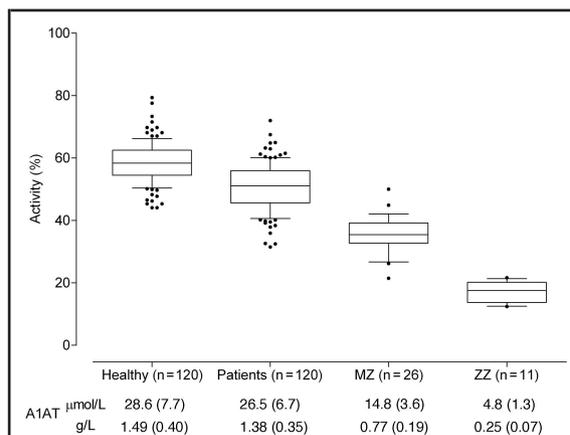
We retained TNBSA in huge excess as a chromophore (16, 22), because in an alkaline medium TNBSA binds to positively charged amino acids, such as lysine, arginine, and histidine.

The within-run assay imprecision was good, with antitryptic capacity (%) measured at 63.4% (1.9%) inhibition for the high level, 44.7% (2.5%) for the medium level, and 12.6% (2.0%) for the low level. The respective CVs were 3.1%, 5.5%, and 16.2% of the measured inhibition. The between-run imprecision over a 20-day period was determined to be 7.5%. These results are consistent with those of the authors that we have previously mentioned [CVs% from 3.6–13.8 (9, 12)].

The limit of detection (LOD) was evaluated by the formula:  $LOD = m + k \times SD$  (23), where  $m$  is the mean and  $SD$  is the  $SD$  of the blank assayed 10 times, and  $k$  a variable depending on the number of steps involved, varying from 2.335 (simple reaction) to 5.185 (complex reaction) for the 5% risk. When  $k = 2.335$ ,  $LOD = 5.0\%$  inhibition; when  $k = 5.185$ ,  $LOD = 9.5\%$  inhibition, which is the value we chose to be on the safe side.

When a single patient sample was diluted, and observed values were compared to expected values, the linearity was found to be:  $y = 1.1075x - 3.7814$ , and  $r^2 = 0.9971$  (Fig. 1). The inaccuracy in antitryptic activities below 10% was expected, given the  $LOD$  of 9.5% we established.

**Reference values.** Whole mean (SD) antitryptic activity determined for the group of apparently healthy refer-



**Fig. 2.** Dependence of the A1AT activity on the pathology (Patients PiM) of the phenotype (PiMZ, PiZZ).

The activity of A1AT significantly differed between the 4 classes ( $P < 0.0001$ , Kruskal–Wallis test;  $P < 0.001$  Dunn multiple posthoc test). The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles.

ence individuals (Healthy PiM,  $n = 120$ ) was 58.4% (6.7%) inhibition.

#### CLINICAL EVALUATION

When we used the Kruskal–Wallis test to comparing the antitryptic activity of the 120 Healthy PiM individuals [mean (SD) activity = 58.4% (6.7%) inhibition] with that of the 156 patients (Fig. 2), we found, as expected, that the Patients PiZZ and the Patients PiMZ groups [ $n = 11$ , activity = 17.2% (3.3%) inhibition and  $n = 26$ , activity = 35.51% (5.9%) inhibition, respectively] displayed a significantly lower mean inhibitory activity than the Healthy PiM group and the Patients PiM group ( $P < 0.0001$ ). The antitryptic activity in the Patients PiM group with various pathologies potentially related to A1AT [ $n = 120$ , activity = 50.7% (7.5%) inhibition] was significantly less than in the Healthy PiM group ( $P < 0.0001$ ), regardless of the patients' underlying pathologies [pulmonary,  $n = 68$ , activity = 51.8% (7.4%) vs other pathology,  $n = 52$ , activity = 49.5% (7.6%)]. On the other hand, we observed no differences based on underlying pathology in the mean activities for patients in the Patients PiM group.

The correlation between the A1AT concentration measured by the immunoassay and the antitryptic activity was positive in the Healthy PiM group, with  $r^2 = 0.78$  and 0.52 in the Patients PiM group, suggesting that some of the measured A1AT protein may be inactive.

In studies of the effects of polymerization, the characteristic bands of A1AT were not detected by isoelectric focusing in the 4 samples that were polymerized by heating, and antitryptic activity was not detectable.

Use of this measurement technique on BAL fluids showed that a sample volume of 50  $\mu$ L was necessary to optimize the measurement. Under these conditions, 20% of the 70 BAL fluids studied presented a detectable antitryptic activity of 23% (13%) inhibition. Given the random recruitment of the patients studied, a study on individuals selected according to pulmonary status should be considered.

## Discussion

Following the recommendations of the WHO (24), the AAT Deficiency Detection Centre (Salt Lake City, UT) has suggested that a program for detecting A1AT deficiencies (25) based on the measurement of antiprotease activity should be developed, implicitly suggesting the need for such a test. The technique we describe here potentially could be applied in this capacity once it has been further validated.

The activity of A1AT, rather than its protein concentration, may be a critical factor in its protective role. In the case of the PiZ phenotype, an 80% reduction of A1AT concentration leads to a collapse of activity, suggesting that this structural alteration (i.e., polymerization) severely affects activity. Antitryptic activity was also lost in the 4 samples in which we polymerized the A1AT with heat treatment. In some rare cases of major A1AT deficiency, antitryptic activity is normal and there are no observed physiological disorders. In such cases, the measurement of antitryptic activity is undoubtedly an improvement over phenotyping and genotyping. Intravenous augmentation therapy is possibly not pertinent in some patients with low A1AT concentration and subnormal activity. What is really important in evaluating this deficiency is not the concentration of A1AT but its antiprotease activity. Moreover, our method for measurement of A1AT activity might allow detection of PiNULL patients who demonstrate possible compensation for A1AT deficiency by activation of other serpins such as  $\alpha$ 1-antichymotrypsin or  $\beta_2$ -microglobulin.

It is notable that 24 of the 120 Patients PiM in our study with A1AT concentrations within the reference interval and a PiM phenotype had significantly lower activities than the standard values [42.13% (2.31%) vs 58.44% (6.66%);  $P < 0.001$  Kruskal-Wallis test]. This result suggests a possible link between their pathology in these patients and low A1AT activity caused by something other than a change in protein concentra-

tion. This result reinforced our interest in developing a routine method for measuring the antitryptic activity.

In an Italian study performed on 2922 subjects, Ferrarotti and coworkers (26) noted that 11% of PiZZ, 38% of PiSZ, and 42% of PiMZ bearers were in good health. They did not evaluate antitryptic activity, but it is conceivable that the good health observed in these patients was more related to their net A1AT activity than to changes in the actual concentrations of A1AT.

In addition, these investigators studied some rare phenotypes of A1AT deficiencies that were detectable only by sophisticated analysis. These investigators noticed that all of these patients suffered from chronic obstructive pulmonary disease. In such cases, the simple measurement of antitryptic activity might be sufficient.

In this study, fewer than 5% of the patients with emphysema displayed low A1AT concentrations, but their A1AT activity, which would have been valuable information to know, was not documented. In this respect, it is interesting that Kalsheker and coworkers (27) may have identified DNA variants of the human *SERPINA1* gene that are not detected by isoelectric focusing but are nevertheless related to pulmonary disease. In particular, the G1237A mutation (G $\rightarrow$ A at 1237 on intron 2; PiEE) has a frequency that was estimated to be 10% by Brantly and coworkers (28) and 13% in the Italian population (29) but seems to be significantly higher among patients suffering from chronic obstructive pulmonary disease and bronchiectasis (30). The concentration of A1AT in people carrying this mutation is normal, and therefore, the measurement of antitryptic activity in the plasma of these patients should be informative and worth testing. This analysis would also be useful for carriers of the PiM phenotype, which is regarded as normal but is sometimes associated with the appearance of chronic obstructive pulmonary disease (31). Conversely, the G1237A mutation has been shown beneficial to patients suffering from cystic fibrosis (32), and effect that could be related to a sustained tryptic activity unchecked by A1AT.

In some viral diseases, such as hepatitis and AIDS, serum A1AT concentrations reach very high values. Bristow and coworkers (33) found circulating A1AT concentrations of 307 (83)  $\mu$ mol/L [16.00 (4.34) g/L], among AIDS patients. These investigators also showed that most of this A1AT was bound to IgG, rendering it inactive, and that the active portion was equivalent to 39 (30)  $\mu$ mol/L [2.03 (1.55) g/L], about one-eighth of the measured circulating concentrations. Again, this example demonstrates how important the measurement of the actual antitryptic activity can be.

A1AT concentration measurement, genotyping, and phenotyping are 3 methods for studying A1AT de-

iciencies. However, none of these methods is fully satisfactory, owing to ethical problems (34), insufficient predictivity, or difficulty of performance. Therefore, we think that our new approach will prove to be a useful method to explore A1AT deficiency, especially in large-scale studies, and that it can complement genetic counseling about risk of future lung disease.

Measurement of the antitryptic capacity in BAL fluids could prove worthwhile, because this information could contribute to the assessment of an eventual attack on the pulmonary mucous membrane.

In conclusion, we have developed a stable, reliable, easily automated assay that could prove very valuable in the overall assessment of A1AT deficiency. Our goal was not to develop an assay competing with phenotyping/genotyping and quantification of A1AT but to offer a functional assay to complement the information from existing tests to explore deficiency of A1AT. Further evaluation on selected populations, particularly

patients with lung or liver diseases, is needed to fully confirm the value of this assay.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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