e-Appendix 1

α₁-antitrypsin polymerizes in alveolar macrophages of smokers with and without α₁-antitrypsin deficiency

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METHODS

Subject characteristics

We collected peripheral lung tissue from 57 patients undergoing lung transplantation for severe emphysema or lung resection for solitary peripheral nodules. In particular, 33 patients were transplanted for severe emphysema, of whom 9 had AAT deficiency (COPD with AATD) and 24 had normal AAT levels (usual COPD). Twenty-four patients were operate for solitary peripheral nodules of which 11 were smokers (smokers without COPD) and 13 were non-smokers. Each patient underwent: interview, electrocardiography, routine blood tests and pulmonary function tests. In subjects displaying an abnormal α1 globulin band at protein electrophoresis, plasma AAT levels were measured by radial immunodiffusion or nephelometry, considering normal range values of 190-350 mg/dl or 90-200 mg/dl respectively. Deficiency was confirmed by genotyping or phenotyping. The phenotype was determined by isoelectric focusing analysis on agarose gel with specific immunological detection [E1]. Genotyping was performed on DNA isolated from paraffin-embedded tissue samples using a commercial extraction kit (QIAamp DNA FFPE tissue kit, Qiagen). Genotyping for detection of the S and Z variants was performed by PCR with fluorescently labelled Taq-Man probes (Vic or Fam labels) on a LigthCycler480 (Roche Diagnostics), as previously reported [E2]. For sequence analysis, all coding exons (II-V) of the AAT gene (SERPINA1, RefSeq: NG_008290) were sequenced, as previously described [E3], using the CEQ 8800 genetic analysis System (Beckman Coulter).

Tissue processing

All the explanted lungs were processed following the same protocol; in particular, there were no differences in sampling procedures in explanted lungs from patients with AAT deficiency (COPD with AATD) or "usual" COPD. Briefly, immediately after harvesting, the lungs were gently fixed in 10% phosphate-buffered formalin (pH 7.2) by airway perfusion, and were cut to obtain parasagittal slices. In particular, large thin blocks (approximately 30 × 25 mm) were cut from the subpleural

areas. In patients who underwent lung resection for nodules, three to six randomly selected tissue blocks were taken from the subpleural parenchyma avoiding areas affected by tumor.

After dehydration all tissue samples were processed for sectioning and embedded in paraffin wax.

Histochemistry

Briefly, after dewaxing and hydration, sections were treated with specific Periodic Acid-Schiff protocol to identified PAS + inclusions representing protein polymers. (E4)

Immunohistochemistry

Immunohistochemistry was carried out by using the following antibody panel: IR505 (recognizing both native and polymerized AAT; 1:100), CD20 (1:100), CD4 (1:20), CD8 (1:50), and neutrophil elastase (1:150) (all antibodies provided by Dako, Santa Barbara, CA, U.S.A.). Intracellular AAT polymers were recognized with the specific monoclonal antibody 2C1 provided by prof. D. Lomas (E5). For all immunohistochemical experiments, negative controls were obtained by incubation of the sections with the omission of primary antibody and using the antibody diluents alone or the appropriate non-immune IgG in each case. Briefly, after dewaxing and hydration, sections were incubated in citrate buffer 5 mM at pH 6.0 in a microwave oven for 30 minutes, for antigen retrieval. Afterwards, sections were treated with rabbit serum (X0901, Dako, Glostrup, Denmark) and incubated for 60 min with the primary monoclonal antibodies. Sections were subsequently incubated with streptavidin-biotin complex conjugated to alkaline phosphatase (Strept AB complex/AP, K0391; Dako, Glostrup, Denmark) for 30 min. Immunoreactivity was visualized with diamino benzidyne (DAB, Dako, Glostrup, Denmark). Finally, the sections were counterstained with Mayer's haematoxylin.

To quantify PAS positive alveolar macrophages, AAT positive alveolar macrophages and AAT polymerized positive alveolar macrophages, at least 20 to 40 non consecutive high-power fields (HPF) and at least 100 macrophages inside the alveolar spaces were evaluated for each patient; results were expressed as percentage of PAS, AAT and AAT polymerized positive macrophages over the total number of macrophages examined (E6). Alveolar macrophages were defined as mononuclear cells with a well-represented cytoplasm, present in the alveolar spaces.

Inflammatory cells (CD20, CD4, CD8, and neutrophil elastase) were evaluates in the alveolar walls. Briefly, 10 to 15 fields, distributed randomly across the slide, were evaluated for each subject and the result was expressed as the number of positive cells·mm−1 of alveolar wall as previously described (E7).

Statistical analysis

All cases were coded and the measurements were made without knowledge of clinical data to avoid observer bias. Differences between groups were evaluated with either ANOVA (for the clinical data) or the Kruskal-Wallis test (for the morphological data). When significant differences among groups were observed comparison between two groups was performed using Student t tests (for the clinical data) or Mann-Whitney U tests (for the morphological data). Correlation coefficients were calculated using the nonparametric Spearman rank method. P values of 0.05 or less were considered to indicate statistical significance. In a subgroup of patients, the same measurements were performed again by the same observer to assess the intraobserver reproducibility, and by a second observer to assess the interobserver reproducibility. The intraobserver correlation coefficiecient for AAT, PAS, and AAT polymerized ranged from 0.90 to 0.94, while the interobserver correlation coefficient ranged from 0.88 to 0.90.
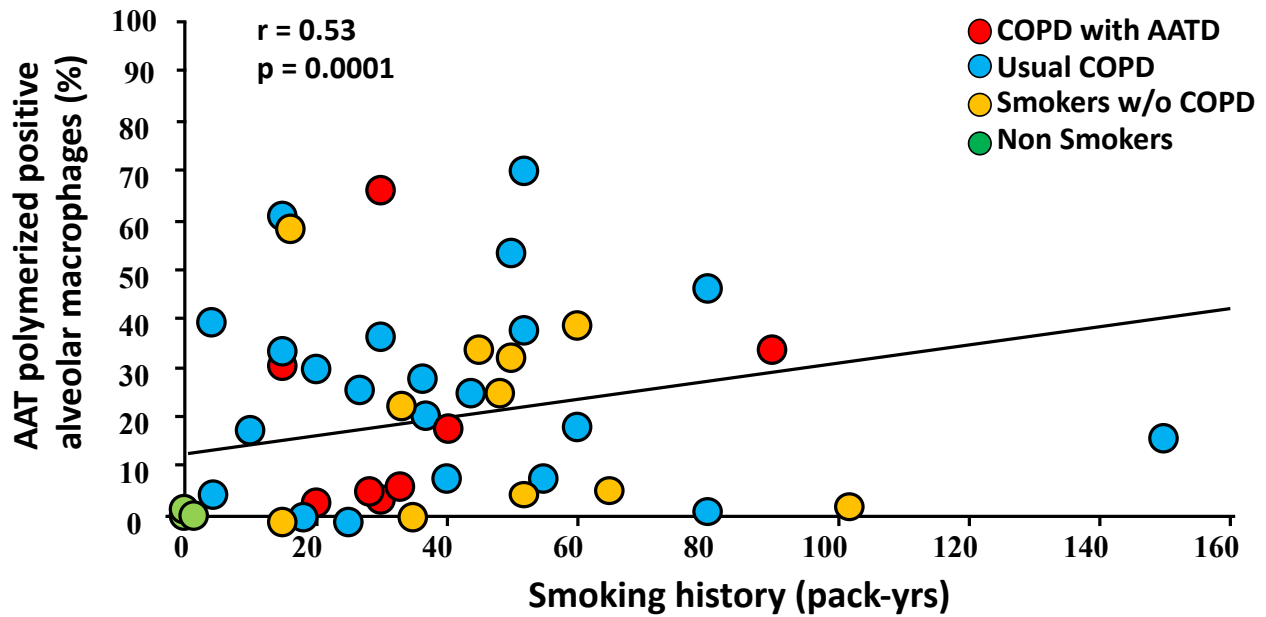
RESULTS

Correlation

The cumulative exposure to cigarette smoke was positively correlated to the percentage of macrophages showing PAS+ inclusions (r=0.41; p=0.003) and those positive for AAT polymers (r=0.53; p=0.0001; e-Fig 1). When we examined the relationship between the presence of polymerized AAT in alveolar macrophages and the lung pathology we found that the percentage of polymerized alveolar macrophages correlated significantly with the emphysema score (r=0.55;p=0.002), the small airway disease score (r=0.44;p= 0.004;e-Fig 2), and the numbers of neutrophils (r=0.31;p=0.05) and also with the number of CD8+T lymphocytes (r=0.51;p=0.002;e-Fig 3). Furthermore, the percent of polymerized AM was inversely correlated with pulmonary function parameters (FEV1: r=-0.44;p=0.002 and FEV1/FVC: r=-0.41;p=0.005).

When these analyses was limited to smokers with and without COPD, no correlations remain significant.

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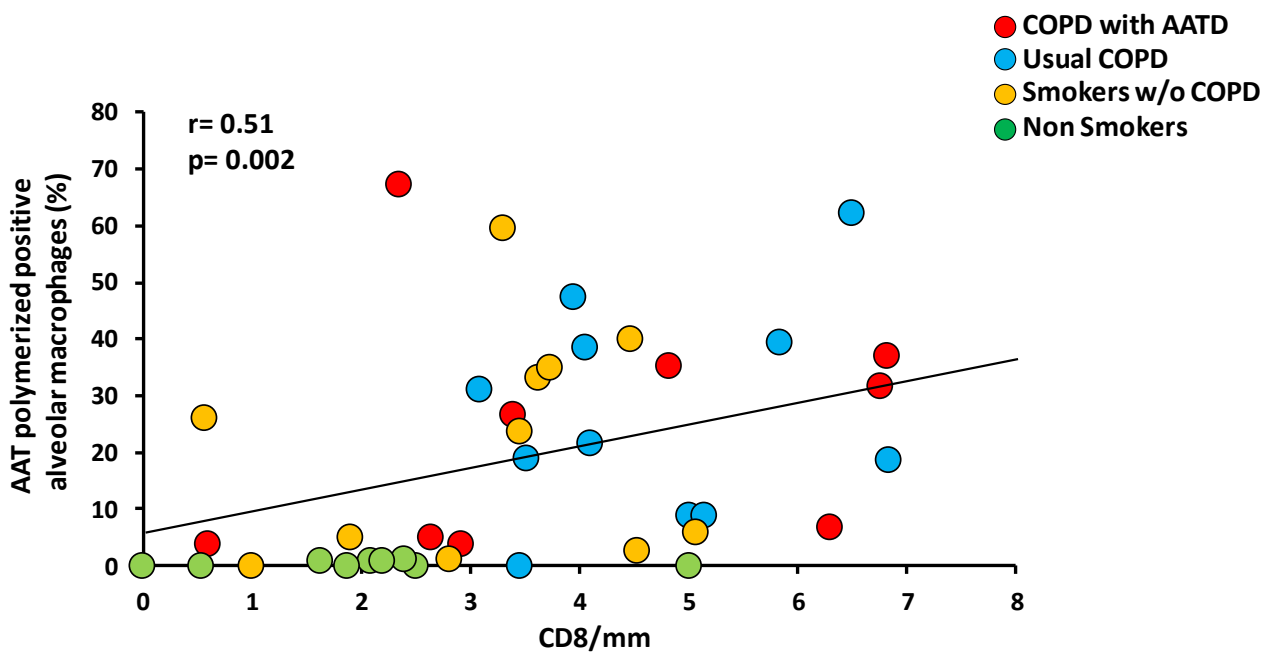
e-Figure 1

e-Figure 1: Correlation between smoking history (pack-years) and percentage of alveolar macrophages positive for AAT polymerized The cumulative exposure to cigarette smoke was positively correlated to the percentage of alveolar macrophages positive for AAT polymerized in the study cohort. Red dots represent COPD patients with AATD patients; blue dots represent patients with usual COPD, yellow dots represent smokers without COPD and green dots represent non-smokers. Spearman's rank correlations: r=0.53, p=0.0001.

e-Figure 2
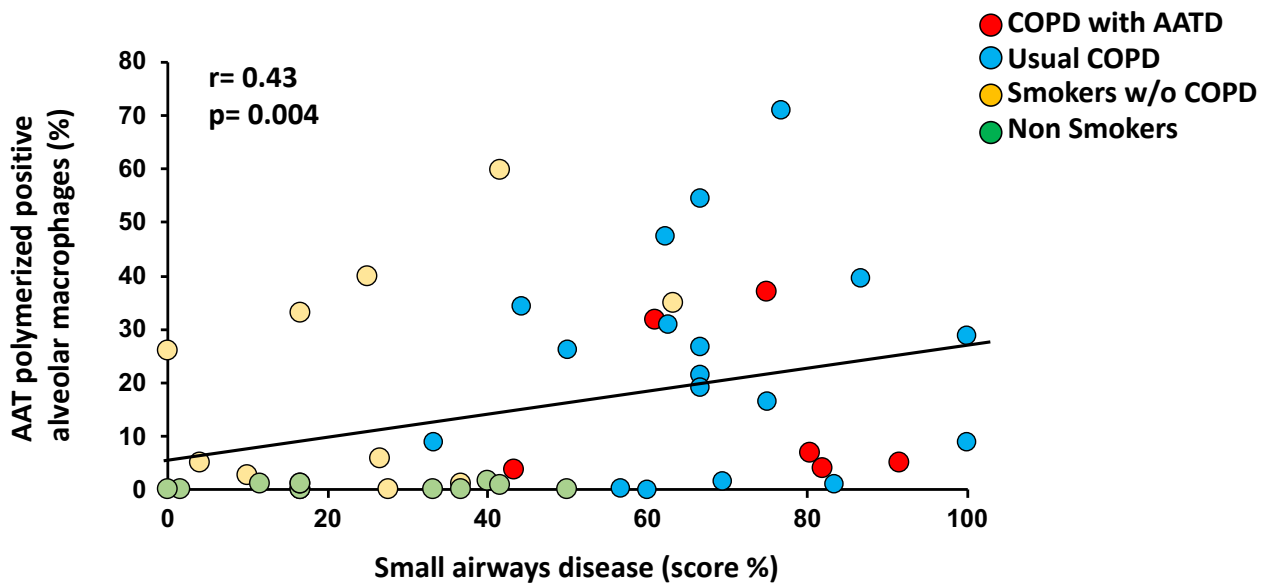
e-Figure 2: Correlation between score of small airways disease (%) and percentage of alveolar macrophages positive for AAT polymerized. The score of small airways disease was positively correlated to the percentage of alveolar macrophages positive for AAT polymerized in the study cohort. Red dots represent COPD patients with AATD patients; blue dots represent patients with usual COPD, yellow dots represent smokers without COPD and green dots represent non-smokers. Spearman's rank correlations: r=0.43, p=0.004.

e-Figure 3

e-Figure 3: Correlation between number of CD8/mm of alveolar walls and percentage of alveolar macrophages positive for AAT polymerized. The number of CD8+ T cells in the alveolar walls was positively correlated to the percentage of alveolar macrophages positive for AAT polymerized in the study cohort. Red dots represent COPD patients with AATD patients; blue dots represent patients with usual COPD, yellow dots represent smokers without COPD and green dots represent non-smokers. Spearman's rank correlations: r=0.51, p=0.002.