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

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Running Title: AAT Polymerization in alveolar macrophages

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Abbreviations: AAT= α1-antitrypsin; AM= Alveolar Macrophages; AATD= α1-antitrypsin
deficiency; COPD = Chronic Obstructive Pulmonary Disease; Glu= glutamic acid; Lys= lysine;
ABSTRACT

Background. The deficiency of α₁-antitrypsin (AAT) is secondary to misfolding and polymerization of the abnormal Z-AAT in liver cells and is associated with lung emphysema. Alveolar macrophages (AM) produce AAT, however it is not known if Z-AAT can polymerize in AM, further decreasing lung AAT and promoting lung inflammation.

Aims. To investigate if AAT polymerizes in human AM and to study the possible relation between polymerization and degree of lung inflammation.

Methods. Immunohistochemical analysis with 2C1 monoclonal antibody specific for polymerized AAT was performed in sections of: 9 lungs from individuals with AAT deficiency (AATD) and severe COPD, 35 smokers with normal AAT levels of which 24 with severe COPD and 11 without COPD, and 13 non-smokers. AM positive for AAT polymers were counted and expressed as percentage of total AM in lung.

Results. AAT polymerization was detected in [27(4-67)]% of AM from individuals with AATD but also in AM from smokers with normal AAT with [24(0-70)]% and without [24(0-60)]% COPD, but not in AM from non-smokers [0(0-1.5)%] (p<0.0001). The percentage of AM with polymerized AAT correlated with pack-years smoked (r=0.53,p=0.0001), FEV₁/FVC (r=-0.41,p=0.005), Small Airways Disease (r=0.44,p=0.004), number of CD8+T-cells and neutrophils in alveolar walls (r=0.51,p=0.002; r=0.31,p=0.05 respectively).

Conclusions. Polymerization of AAT in alveolar macrophages occurs in lungs of individuals with AATD but also in smokers with normal AAT levels with or without COPD. Our findings highlight the similarities in the pathophysiology of COPD in individuals with and without AATD, adding a potentially important step to the mechanism of COPD.

Key words: COPD, emphysema, serpins, cigarette smoking
INTRODUCTION

\( \alpha_1 \)-antitrypsin (AAT) is the archetypal member of the serine protease inhibitor (SERPIN) superfamily. Severe deficiency of this protein, secondary to an inherited disorder, is linked to the development of early onset emphysema. About 95% of the significant clinical deficiency is caused by the Z variant of the protein that results from the substitution of a glutamic acid (Glu) by a lysine (Lys) at position 342.\(^{1-5}\) Approximately 0.06% of individuals of North European descent have severe deficiency of AAT with plasma levels of less than 0.2 g/L.\(^{1-5}\) The Glu to Lys substitution in Z-AAT results in abnormal protein folding with in the endoplasmic reticulum (ER) of the hepatocyte, protein polymerization and intracellular retention with consequent low AAT serum levels.\(^{1-5}\) Thus the effect of the Z mutation is not a failure of synthesis (Z-AAT is processed normally until it reaches the final stage of the hepatocyte ER pathway), but a failure in folding and secretion. About 85% of the Z-AAT is removed by ER-associated degradation or aggregates to form polymers, while 15% is secreted in the serum.\(^{1-6}\)

Polymerization of Z-AAT in the liver causes a “toxic gain of function” within hepatocytes\(^3\), with ER stress and activation of NF-\( \kappa \)B\(^{7-9}\) triggering an inflammatory reaction in response to protein misfolding and polymerization in the hepatocytes that predisposes to neonatal hepatitis and liver cirrhosis.\(^{10,11}\)

Epithelial barrier macrophages such as alveolar macrophages, intestinal and epithelial macrophages and breast milk macrophages, along with blood monocytes, are also important producers of AAT in their local milieu.\(^{12-14}\) To a minor extent other cells in the lung including lung epithelial cells, bronchial epithelial cells (BECs), endothelial cells, and the human A549 cell line of alveolar epithelial cells, as well as polymorphonuclear leukocytes and neutrophils, have been found to also produce AAT.\(^{15-19}\) Alveolar macrophages develop from fetal liver under the control of GM-CSF in the first days of life, paralleling the development of the alveoli and then maintain themselves by in situ self-renewal.\(^{20-22}\) Perhaps, due to their different origin, there is an important difference in production of AAT between blood monocytes (which produce three fold less AAT) and alveolar macrophages\(^{14}\), suggesting that alveolar macrophages are preprogramed by their liver origin or that, once in the lung milieu, they up-regulate AAT gene expression.

Alveolar macrophages can produce relatively large amounts of AAT directly into the lung but, as with hepatocytes, the production and secretion of AAT is regulated by inflammatory mediators such as Lipopolysaccharides (LPS) and the acute phase cytokine Interleukin IL-6. The synthesis of AAT is also modulated by the presence of elastase in a dose and time dependent way.\(^{23}\) Under these stimuli wild type PiMM AAT monocytes can increase the synthesis and secretion of AAT by up to 10 fold.\(^{14}\)
It would seem that the normal production of AAT by alveolar macrophages, potentially increased under the modulation of inflammatory mediators and elastase, could well polymerize in the ER of alveolar macrophages in PiZZ individuals, a possibility that has never been studied in human lung tissue. If that were the case, AAT polymerization in alveolar macrophages will not only contribute to loss of AAT function due to diminished secretion in the alveoli, but also, as in the liver, to “toxic gain of function” with all its complex and detrimental consequences.

It was the aim of our study to assess whether alveolar macrophages in the lung tissue from individuals with PiZZ AAT deficiency formed AAT polymers and if polymerization could be related to inflammation within the lung. For this purpose, we studied lung sections from individuals with COPD with AAT deficiency undergoing lung transplantation and compared them with lungs of smokers with COPD and normal AAT (“usual” COPD), smokers without COPD, and non-smokers. The results of this investigation have been presented in abstract form.²⁴
METHODS

Subject Characteristics

We studied the tissues from the lungs of 33 patients undergoing lung transplantation for severe COPD: 9 had PiZZ α1-antitrypsin deficiency (COPD with AATD) and 24 had normal levels of AAT ("usual" COPD). AATD was confirmed by serum levels, together with genotyping/phenotyping in all cases. Sections from the lungs of 11 smokers with normal lung function and 13 non-smoking subjects, who had lung resection for solitary nodules, were included for comparison. All 57 subjects underwent pulmonary function tests prior to surgery and provided informed written consent. The study conformed to the Declaration of Helsinki. All aspects of this study were approved by the local Ethics Committee (reference number 0006045). Details are reported in the Online Supplement.

Histochemistry, immunohistochemistry and morphometric analysis

Lung tissue preparation, histochemistry and immunohistochemistry were performed as previously described and detailed in the Online Supplement.25,26 The lung tissue specimens were fixed in formalin, embedded in paraffin wax and cut. At least three lung sections per case were stained with periodic acid-Schiff (PAS) and immunostained according to the standard peroxidase-antiperoxidase method with a commercial polyclonal anti-AAT antibody recognizing total AAT (both native and polymerized, IR505 Dako, Denmark) and with the specific monoclonal antibody 2C1 that recognizes intracellular AAT polymers but not native (monomeric), reactive loop cleaved or latent AAT.27 Negative controls for nonspecific binding were processed either omitting the primary antibody or using isotype IgG and revealed no signal.

To quantify AAT positive alveolar macrophages, PAS positive inclusions in 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 subject. The results were expressed as percentage of positive macrophages over the total number of macrophages examined.25,26 Alveolar macrophages were defined as mononuclear cells with a well-represented cytoplasm, present in the alveolar spaces.

As positive control for AAT polymer staining we examined 6 liver samples from PiZZ patients who underwent liver transplantation related to AATD. 5 µm sections were stained with PAS and the specific monoclonal antibody 2C1 to detect AAT polymerization, following the same protocol used for pulmonary tissue.

Neutrophils, macrophages, T CD4+ lymphocytes, T CD8+ lymphocytes and B lymphocytes were identified by immunohistochemistry and counted in the alveolar walls in order to evaluate a
possible correlation between AAT (native and polymerized) and the degree of lung inflammation. Details are reported in the Online Supplement.

Using the semi quantitative method described by us we assessed the Small Airways Disease score (inflammation, muscle, wall thickness) in all airways less than 2 mm in diameter. Each of bronchiole 2 mm and less in diameter was examined separately for the presence of inflammatory cell infiltrate, smooth muscle hypertrophy and wall thickness. For each airway, a score from 0 (normal) to 3+ (most abnormal) was assigned for each pathological feature. Scores for individual features were summed and expressed as percentage of maximal possible score.

A macroscopic quantification of emphysema was performed in all explanted lungs, using the method of Heard and colleagues. Because lungs were not fixed in inflation at a constant pressure, we were not able to use mean linear intercept (Lm) for the microscopic quantification of emphysema (air space size). We instead undertook a semiquantitative score of the extent of microscopic emphysema (0,1,2,3+) in every slide available in all cases and expressed this as percentage of the maximal possible score.

The possible relationship between AAT polymerization and inflammatory response was also examined in liver tissue. From each liver surgical sample, two consecutive sections of 5 µm thick were cut and stained with 2C1 antibody to identify polymers in one section (following the same protocol used for pulmonary tissue) and with CD45 antibody to identify total leukocytes in the other consecutive section. An intensity score from 0 to 3 for the extent of polymerization and of CD45 positive cells was graded in 50 fields for each slide pair.

All analyses were performed using a Leica light microscope and video recorder linked to a computerized image analysis system (Leica LAS w3.8).

**Statistical analysis**

Group differences were evaluated by analysis of variance (ANOVA) and unpaired Student t test for clinical data, and by Kruskal–Wallis test and Mann–Whitney U test for morphological data. Correlation coefficients were calculated by the Spearman rank method. P values of 0.05 or less were considered to indicate statistical significance. Details are reported in the Online Data Supplement.
RESULTS

Clinical Characteristics

Nine patients transplanted for severe COPD had low serum AAT levels consistent with severe AATD and confirmed by either genotyping or phenotyping (8 ZZ and 1 ZI). All patients with “usual” COPD, smokers without COPD and non-smokers had a normal α1 band on protein electrophoresis.

The clinical characteristics of the subjects in this study are shown in Table 1. There were no differences in age and amount smoked (14% current smokers and 86% recent ex-smokers). The values of FEV1 (% predicted) and FEV1/FVC (%) were similarly decreased in the COPD with AATD and in “usual” COPD, whereas they were in the normal range in smokers without COPD and non-smokers.

Histochemical and immunohistochemical findings

Positive staining with anti-AAT antibody IR505, which stains both native and polymerized AAT, was observed mainly in alveolar macrophages (AM) and occasionally in the alveolar walls (Fig.1 panels A-B). There was no significant difference in the percentage of alveolar macrophages positive for total (native and polymerized) AAT between: COPD with AATD, “usual” COPD, smokers without COPD and non-smokers (Fig.1, C).

The percentage of PAS positive AM was increased not only in individuals with AATD, but also in smokers with or without COPD and normal AAT levels compared to non-smokers, where no PAS positive intracellular inclusion were seen (Fig.2). Furthermore, the percentage of periodic acid-Schiff (PAS) positive AM was also increased in smokers with “usual” COPD compared to smokers without COPD (Fig. 2). The PAS inclusions were similar to those seen in the liver from individuals with PiZZ AATD (Fig.3 A-B). The use of the polymer specific 2C1 monoclonal antibody (recognizing specific intracellular AAT polymers) showed a similar pattern for polymerization in AM and in liver sections of PiZZ AAT individuals (Fig.3 C-D). The percentage of AM that stained positive for polymers was increased not only in individuals with AATD, but also in smokers with or without COPD and normal AAT levels compared to non-smokers, where no polymerization was seen (Fig.4).

When all cases were considered together, the cumulative exposure to cigarette smoke (packs/year) 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).
The score for Small Airways Disease in COPD subjects with and without AATD was significantly higher than that in smokers without COPD and in non-smokers (Table 2).

On macroscopic analysis both transplanted groups (with and without AATD) had severe diffuse emphysema with marked extension of lung destruction in both upper and lower lobes. The extent of lung destruction made it impossible to define the type of macroscopic emphysema (Centrilobular or Panlobular). The semi quantitative score of the extent of microscopic emphysema showed that subjects with COPD, with and without AATD, had an increased emphysema score when compared with both smokers without COPD and non-smokers (Table 2).

The number of lymphoid follicles/cm² in COPD subjects with and without AATD were significantly higher than in smokers without COPD and in non-smokers (Table 2), as were the number of B, CD4+ and CD8+ lymphocytes in the alveolar wall (Table 2).

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), the number of neutrophils (r=0.31; p=0.05) and CD8+ lymphocytes in the alveolar walls (r=0.51; p=0.002; e-Fig 3). Furthermore, the percent of polymerized AM was inversely correlated with pulmonary function (FEV₁: r=-0.44; p=0.002 and FEV₁/FVC: r=-0.41; p=0.005).

In liver tissue there was a positive correlation between the score of polymerization and that of infiltration of inflammatory cells (CD45) (r=0.56; p<0.0001).
Discussion

Alveolar macrophages are highly prevalent within the lung and can produce considerable amounts of AAT. We investigated if polymerization due to misfolding, aggregation and retention of abnormal Z-AAT that takes place in liver cells, could also occur in human alveolar macrophages. Our results showed that AAT polymers are present in alveolar macrophages in the lung of individuals with PiZZ AAT deficiency (COPD with AATD). Surprisingly, we also found AAT polymers in alveolar macrophages of smokers with COPD and normal AAT levels (“usual” COPD) and in smokers without COPD, but not in non-smokers.

The presence of significant polymerization of AAT in alveolar macrophages directly in human lung tissue had never been previously reported. Alveolar macrophage polymers may be a source of the bronchoalveolar lavage (BAL) polymers previously described in individuals with PiZZ AAT deficiency.\textsuperscript{30} We have found that periodic acid-Schiff (PAS) positive granules can be seen in alveolar macrophages by light microscopy. With the use of a specific antibody we showed that the PAS positive granules present in both PiZZ and PiMM AAT alveolar macrophages are, at least in part, due to AAT polymerization. There was a large variation in the percentage of macrophages showing AAT polymerization (ranging from 0 to 55%), possibly because some polymers might be too small to be detected (polymers can vary in size from 2 to many molecules which can aggregate to form the visible granules).\textsuperscript{31} In addition, this variation could also depend on the alveolar macrophages phenotypes and their proportion in the lung, since anti-inflammatory M2 macrophages have been shown to express higher AAT mRNA, and thus potentially more polymerization, than pro-inflammatory M1 macrophages.\textsuperscript{32}

AAT contribution by alveolar macrophages.

The polymerization of AAT within lung alveolar macrophages can have severe consequences for lung homeostasis and the development of emphysema associated with AAT deficiency. Liver produces wild-type M-AAT that diffuses through the endothelial barrier of the lung providing alveolar concentration of 10-15% of the plasma AAT level,\textsuperscript{33-35} and this concentration would be significantly supplemented by the secretion of AAT from alveolar macrophages.\textsuperscript{14} It has been calculated that there are approximately 20x10\textsuperscript{9} lung alveolar macrophages which produce three times more AAT than bone marrow derived circulating monocytes,\textsuperscript{14} either because they are already programed in the fetal liver, or because they are reprogramed by the lung micro-environment promoting the more efficient and/or increased production. The fact that alveolar macrophages reside directly at the site where AAT functions as an antiprotease and modulator of
inflammation, suggests a specific differentiation of these cells and highlights their important contribution to the maintenance of lung homeostasis and its failure in deficient states.

Mechanisms of AAT polymerization in the lung.

It has been clearly demonstrated that under stimulation PiMM and PiZZ alveolar macrophages produce similar AAT mRNA levels,\(^{14}\) however PiZZ alveolar macrophages produced 10 times less AAT protein than PiMM alveolar macrophages. This suggests that the defect is at the secretory level, and that the secretory defect secondary to protein misfolding and polymerization seen in the liver, is also present in alveolar macrophages. Unexpected was the finding, never reported before, of AAT polymers in the alveolar macrophages of smokers with COPD and normal AAT levels (“usual” COPD) and also in smokers without COPD, but not in non-smokers. All inhibitory SERPINs can be induced to polymerize by high temperature, oxidation and incubation with denaturants.\(^{31}\) These agents perturb the structure of AAT, opening β-sheet A-sheet to allow polymerization, although the rate of polymer formation is slower in wild-type M than mutant Z AAT. It has been shown that cigarette smoke can greatly accelerate PiZ-AAT polymerization and oxidize PiM-AAT in mice and human plasma\(^ {36}\) that is in keeping with the association between cigarette smoking and polymerization reported in our study. This may explain our novel finding of AAT polymers present in alveolar macrophages from smokers with normal levels of AAT.

Possible consequences of AAT polymerization.

The lung disease seen in individuals with PiZZ AAT deficiency is usually thought as secondary to the low levels of circulating liver-produced AAT, to which we can now add the loss of the AAT secreted by the alveolar macrophages due to AAT polymerization. Furthermore, AAT polymerization could also contribute to the mechanism of disease by triggering important pro-inflammatory effects. It has been previously reported that polymers of AAT in BAL from individuals with PiZZ AAT deficiency\(^ {30}\) are chemotactic for human neutrophils in vitro and in mouse models of disease.\(^ {37-39}\) Along with a “loss of AAT function” there may be an additional “toxic gain of function” originating from the accumulation of misfolded and aggregated AAT in alveolar macrophages endoplasmic reticulum (ER), which could induce ‘ER stress’ and the consequent Unfolded Protein Response (UPR) that normally ensures that misfolded proteins are removed for degradation. However chronic ER stress, coupled with cigarette smoking, could tip the UPR from been adaptive to promoting inflammation.\(^ {40}\) Although we have not studied this possibility, the induction of UPR secondary ER stress in blood monocytes from PiZZ AAT individuals\(^ {41}\) and in bone marrow derived macrophages\(^ {42}\) has been shown to potentiate pro-
inflammatory signaling, including the induction of genes encoding CXC-chemokine ligand 1 (CXCL1), CXCL2, TNF, IL-1, and IL-6.41

The following events could plausibly take place in the lungs of smokers with and without AAT deficiency (Fig.5): inflammatory stimuli, cigarette smoke, free elastase and elastase-AAT-complexes would stimulate an increase production of AAT in alveolar macrophages, which could misfold and polymerize in the endoplasmic reticulum causing endoplasmic reticulum stress and activation of the UPR. As in a vicious circle (Fig.5), UPR activation by increasing the production of pro-inflammatory cytokines and chemokines, such as IL-6, would increase the inflammation that will induce further AAT production, further misfolding and retention in macrophages endoplasmic reticulum thus perpetuating the endoplasmic reticulum stress. The correlation between the extent of polymerization and the severity of inflammation in lung and liver is in support of this hypothesis.

Other local factors such as local hypoxia, as seen in COPD, could add to ER stress. Similar mechanisms are thought to play an important role in autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis.43-45

If this were the case, ER stress would be an important added stimulus and contributor to the innate and adaptive immune inflammation that we have described in severe PiZZ AAT deficiency and in “usual” COPD.25 Importantly, ER stress does not always induce inflammation since cellular adaptation to chronic ER stress can also suppress the inflammatory response to unfolded protein (UPR). How cells decide between proinflammatory and anti-inflammatory UPR signaling is poorly understood.46,47 This phenomenon could perhaps explain why AAT polarization is seen in our population of smokers without COPD, who have less lung inflammation.

The findings described emphasize the complex role that could be played by the molecular abnormalities of AAT in the development of COPD and emphysema and highlights another important and potentially damaging effect of cigarette smoking. Our findings also highlight the similarities, ever more evident, in the pathophysiology of COPD in smokers with and without AAT deficiency and add another potentially important step to the complex mechanism underlying the disease.

Conclusion

Polymerization of AAT in alveolar macrophages occurs in the lungs of individuals with AATD but also in smokers with normal AAT levels with or without COPD. Our findings highlight the similarities in the pathophysiology of COPD in individuals with and without AATD, adding a potentially important step to the mechanism of COPD.
ACKNOWLEDGMENTS:

AUTHOR’S CONTRIBUTION:

Conception and design: MGC, MS, DL.
Performing experiments: EB, RB, CR, MT, SB, GT
Clinical characterization: DB, FR, SB, FC, GT, SF, AS
Analysis and interpretation: MGC, MS, DL, EM, EB, MPFB
Drafting the manuscript for important intellectual content: MGC, MS, DL, EM, EB, MPFB


11. Eriksson S, Carlson J, Velez R. Risk of cirrhosis and primary liver cancer in alpha 1-


Table 1: Clinical characteristics of the subjects in the study cohort.

<table>
<thead>
<tr>
<th></th>
<th>COPD with AATD</th>
<th>Usual COPD</th>
<th>Smokers w/o COPD</th>
<th>Non Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of subjects, n</strong></td>
<td>9</td>
<td>24</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td>53±3</td>
<td>57±1</td>
<td>62±2</td>
<td>56±6</td>
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<tr>
<td><strong>Smoking history, pack-years</strong></td>
<td>34±8</td>
<td>41±7</td>
<td>48±7</td>
<td>-</td>
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<tr>
<td><strong>Current/ex-smokers, n</strong></td>
<td>0/9</td>
<td>2/22</td>
<td>4/7</td>
<td>-</td>
</tr>
<tr>
<td><strong>FEV₁, % pred</strong></td>
<td>19±2†</td>
<td>20±2†</td>
<td>98±3</td>
<td>108±5</td>
</tr>
<tr>
<td><strong>FEV₁/FVC, %</strong></td>
<td>35±5†</td>
<td>37±3†</td>
<td>77±2</td>
<td>85±4</td>
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</tbody>
</table>

Definition of abbreviations: AATD = α₁-antitrypsin deficiency; COPD = chronic obstructive pulmonary disease; “usual” COPD = COPD with normal AAT levels;
Values are expressed as the means±SD.
† Significantly different from smokers without (w/o) COPD and non-smokers (p<0.0001).
Table 2: Quantification of lung pathology and inflammation.

<table>
<thead>
<tr>
<th></th>
<th>COPD with AATD</th>
<th>Usual COPD</th>
<th>Smokers w/o COPD</th>
<th>Non Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Airways Disease (score %)</td>
<td>78(43-92)**</td>
<td>67(33-100)**</td>
<td>26(0-63)</td>
<td>17(0-50)</td>
</tr>
<tr>
<td>Emphysema (score %)</td>
<td>83(67-100)**</td>
<td>84(33-100)**</td>
<td>0(0-17)</td>
<td>0(0-0)</td>
</tr>
<tr>
<td>Lymphoid follicles/cm²</td>
<td>4.6(0.7-16.5)**</td>
<td>1.5(0-6.1)**</td>
<td>0(0-2.5)</td>
<td>0(0.0-0.8)</td>
</tr>
<tr>
<td>B cells/mm of alveolar wall</td>
<td>2.1(0-4.4)**</td>
<td>0.9(0-5.0)**</td>
<td>0.2(0-0.63)</td>
<td>0.3(0-0.9)</td>
</tr>
<tr>
<td>CD4⁺ cells/mm of alveolar wall</td>
<td>5.5(0.9-10.8)*</td>
<td>6.1(1.6-11.9)*</td>
<td>2.26(0.2-4)</td>
<td>2.1(0-5.4)</td>
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<tr>
<td>CD8⁺ cells/mm of alveolar wall</td>
<td>3.4(0.6-6.8)§</td>
<td>4.1(3.0-6.8)*</td>
<td>3.4(0.6-5.1)</td>
<td>2.1(0-5.2)</td>
</tr>
<tr>
<td>Neutrophils/mm of alveolar wall</td>
<td>6.3(1.2-15.9)</td>
<td>9.4(4.5-13.9)§</td>
<td>6.8(2.5-9.5)</td>
<td>3.8(0-15.1)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: AATD = a1-antitrypsin deficiency; COPD = chronic obstructive disease; usual COPD: COPD with normal AAT levels.

Values are expressed as median(range).

* or ** Significantly different from smokers without (w/o) COPD and non-smokers (*p<0.05 or **p<0.01)

§ Significantly different from usual COPD (p<0.05)

§ Significantly different from non-smokers (p<0.05)
Figure Legends:

Figure 1. Total (native and polymerized) α1-antitrypsin (AAT) immunostaining in alveolar macrophages. Quantification of AAT expression in alveolar macrophages of patients with chronic obstructive pulmonary disease and α1-antitrypsin deficiency (COPD with AATD), “usual” COPD (COPD with normal AAT levels), smokers without COPD, and non-smokers. (A) Representative examples of AAT expression in the lung of a COPD patient with AATD, and (B) in the lung of a non-smoker. Positive staining (in brown) was mainly observed in alveolar macrophages and occasionally in the alveolar wall. Immunostaining with polyclonal antibody IR505 anti-AAT (A and B). Scale bars = 40 μm. (C) The percentage of alveolar macrophages positive for AAT was not significantly different among the four groups of subjects examined. Horizontal bars represent median values.

Figure 2. PAS staining in alveolar macrophages. Quantification of PAS expression in alveolar macrophages of patients with COPD and α1-antitrypsin deficiency (COPD with AATD), “usual” COPD (COPD with normal AAT levels), smokers without COPD, and non-smokers. (A) Representative examples of PAS expression in the lung of a COPD patient with AATD, and (B) in the lung of a non-smoker. Positive staining (in violet) was mainly observed in alveolar macrophages; arrow indicate PAS positive inclusion. Scale bars = 30 μm. (C) The percentage of PAS positive alveolar macrophages was increased in patients with AATD, and in smokers with and without COPD compared to non-smokers. Furthermore, the percentage of alveolar macrophages positive for PAS was increased in “usual” COPD compared to smokers without COPD. P values in the figure represent Mann–Whitney U tests. Kruskal–Wallis test: p<0.0001. Horizontal bars represent median values.

Figure 3. PAS staining and immunostaining for AAT polymers in liver and lung sections of AATD patients. (A and B) Representative examples of PAS expression in the liver of a patient with AATD (A) and in the lung (B) of a COPD patient with AATD. Positive PAS staining in violet. (C and D) Representative examples of AAT polymers expression in the liver of a patient with AATD (C) and in the lung (D) of a COPD patient with AATD. Positive immunostaining with specific monoclonal antibody 2C1 specific for AAT polymers in brown (C and D). A-C: Scale bars = 30 μm. D: Scale bar = 15 μm.

Figure 4. α1-antitrypsin (AAT) polymers in alveolar macrophages. Quantification of AAT polymers expression in alveolar macrophages of patients with COPD and α1-antitrypsin deficiency
(COPD with AATD), “usual” COPD (COPD with normal AAT levels), smokers without COPD, and non-smokers.

(A) Representative examples of AAT polymers expression in the lung of a COPD patient with AATD and (B) in the lung of a non-smoker. Positive staining (in brown) was mainly observed in alveolar macrophages; arrows indicate AAT positive polymers. Immunostaining with monoclonal antibody 2C1 anti-AAT polymerized (A and B). Scale bars = 30 µm.

(C) The percentage of alveolar macrophages positive for AAT polymerized was increased in COPD patients with AATD, in “usual” COPD and in smokers without COPD compared to non-smokers. P values in the figure represent Mann–Whitney U tests. Kruskal–Wallis test: p<0.0001. Horizontal bars represent median values.

Figure 5. The pathway of lung inflammation induced by AAT polymerization. The inflammatory response induced by smoking would upregulate α-1ATmRNA in alveolar macrophages. This would increase AAT production that could misfold and polymerize in the endoplasmic reticulum (ER) causing ER stress that, with the enhancement of a “second hit” by cigarette smoke, causes activation of the Unfolded Protein Response (UPR). As in a vicious circle UPR activation would further increase the expression of pro-inflammatory genes and lung inflammation, which would induce further AAT production. Furthermore, the chemotactic role of AAT polymers will attract neutrophils further increasing the inflammatory response, all contributing to the worsening of the disease.