Bacterial load and inflammatory response in sputum of alpha-1 antitrypsin deficiency patients with COPD

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Background: Airway inflammation may drive the progression of chronic obstructive pulmonary disease (COPD) associated with alpha-1 antitrypsin deficiency (AATD), but the relationship between airway microbiota and inflammation has not been investigated.

Methods: We studied 21 non-treated AATD (AATD-noT) patients, 20 AATD-COPD patients under augmentation therapy (AATD-AT), 20 cigarette smoke-associated COPD patients, 20 control healthy smokers (CS) and 21 non-smokers (CON) with normal lung function. We quantified sputum inflammatory cells and inflammatory markers (IL-27, CCL3, CCL5, CXCL8, LTb3, MPO) by ELISA, total bacterial load (16S) and pathogenic bacteria by qRT-PCR.

Results: AATD-AT patients were younger but had similar spirometric and DLCO values compared to cigarette smoke-associated COPD, despite a lower burden of smoking history. Compared to cigarette smoke-associated COPD, AATD-noT and AATD-AT patients had lower sputum neutrophil levels (p = 0.0446, p = 0.0135), total bacterial load (16S) (p = 0.0081, p = 0.0223), M. catarrhalis (p = 0.0115, p = 0.0127) and S. pneumoniae (p = 0.0013, p = 0.0001). Sputum IL-27 was significantly elevated in CS and cigarette smoke-associated COPD. AATD-AT, but not AATD-noT patients, had IL-27 sputum levels (pg/ml) significantly lower than COPD (p = 0.0297) and these positively correlated with FEV1/% predicted values (r = 0.578, p = 0.0307).

Conclusions: Compared to cigarette smoke-associated COPD, AATD-AT (COPD) patients have a distinct airway inflammatory and microbiological profile. The decreased sputum bacterial load and IL-27 levels in AATD-AT patients suggests that augmentation therapy play a role in these changes.

Keywords: alpha-1 antitrypsin deficiency, COPD, chronic airway inflammation, respiratory disability, sputum

Introduction

Alpha-1 antitrypsin deficiency (AATD) is a genetic condition considered a risk factor for developing chronic obstructive pulmonary disease (COPD).1 An increased burden of neutrophils is characteristic of AATD lungs.2 AAT, which is coded by the SERPINA1 gene (or Protease inhibitor-PI) is a serine protease inhibitor mainly secreted by the hepatocytes whose major target is elastases but can also inhibit the function of thrombin, trypsin, chymotrypsin, plasmin and plasminogen activator.2

In patients without AATD, neutrophilic pulmonary inflammation is considered central to the development and progression of cigarette smoke-associated COPD.3–5
Neutrophilic inflammation correlates with stable COPD severity, and it further increases during COPD exacerbations. Neutrophil-related inflammatory mediators such as leukotriene (LT)Ba, chemokine (C-X-C motif) ligand 8 (CXCL-8, previously named interleukin-8), chemokine ligand 3 [CCL3 also known as macrophage inflammatory protein-1α (MIP-1α)], and chemokine ligand 5 [CCL5 also known as RANTES (regulated on activation, normal T cell expressed and secreted)] are increased in the lower airways of cigarette smoke-associated COPD patients, particularly in subjects with stable severe disease.

Increased neutrophilia is also associated to increased levels of IL-27 cell expressed and secreted) in exacerbations.

A pathogenic role of bacterial load leading to bronchial inflammation is implicated in cigarette smoke-associated COPD. Augmentation therapy, ie weekly infusion of exogenous AAT, in patients with COPD associated with severe AATD restores AAT levels to normal in blood, sputum and bronchoalveolar lavage (BAL) slowing the progression of the disease, decreasing the number of exacerbations, and sputum neutrophil numbers, LTB4 levels and MPO expression.

The relationship between bacterial load and sputum inflammation in patients with AATD, whether under augmentation therapy or untreated, has not been studied. The aim of the present study was to investigate the total and specific bacterial load in sputum of patients with COPD associated to AATD compared to patients with cigarette smoke-associated COPD and control healthy smokers and non-smokers with normal lung function, and to correlate these data with inflammatory cells, cytokines and physiological parameters.

Methods

Subjects

Twenty cigarette smoke-associated COPD patients, 20 COPD patients with alpha-1 antitrypsin deficiency (AATD) in augmentation therapy (AATD-AT), 21 AATD patients not in augmentation therapy (AATD-noT), 20 control smokers with normal lung function (CS) and 21 control non-smokers with normal lung function (CON) who underwent sputum collection were recruited from the Respiratory Medicine Unit of the Istituti Clinici Scientifici Maugeri’s Institute of Veruno, Institute of Cassano delle Murge, IRCCS Policlinico S. Matteo, Pavia (Italy), and from the Spedali Civili, Brescia (Italy), and Pneumology Unit of Aosta (Italy). COPD was defined, according to international guidelines. Diagnostic criteria for AATD were as from the current ATS/ERS statement. Indications for AT are for patients with severe AATD and compound heterozygotes or homozygotes of deficient/Null variants of SERPINA1 gene (not only PI*ZZ but also PI*SZ, PI*Null/Null etc.) and with a significant airflow obstruction and in the context of general disability and health impairment. In COPD patients and in patients with AATD the severity of the airflow obstruction was graded using current GOLD criteria. COPD and AATD-AT patients had a FEV1/FVC ratio lower than 70% of predicted values at post-bronchodilator spirometry and absence of history and symptoms of asthma. In all patients, the severity of emphysema was graded based on high-resolution computed tomography (HRCT) scan radiologic reports as follows: 0=absence of emphysema, 1=1–25% of lung with emphysema, 2=26–50% of lung with emphysema, 3=51–75% of lung with emphysema, 4=76–100% of lung with emphysema. COPD patients and patients with AATD with evidence of bronchiectasis were excluded. All former smokers had stopped smoking from at least one year before inclusion in the study. All COPD patients and AATD patients were stable with no previous exacerbation in the three months before collection of the sputum. None of the subjects was treated with theophylline, antibiotics, antioxidants, mucolytics, and/or glucocorticoids in the three months prior sputum collection. Cigarette smoke-associated COPD patients and patients with COPD and AATD were using short-acting inhaled β2-agonists (SABA) and/or regular long-acting inhaled β2-agonists (LABA) and/or regular inhaled long-acting anti-muscarinics (LAMA) at the time of their recruitment. AATD-AT patients had been under augmentation therapy for at least three months. Subjects were tested for peripheral blood levels of AAT and for genetic analysis of SERPINA1 gene. Testing was performed on dried blood spot samples at the National Reference Center for AATD at the University of Pavia. The study conformed to the Declaration of Helsinki and was approved by the ethics committee of the Istituti Clinici Scientifici Maugeri SpA, Società Benefit (n. 989 CE). (Protocol Registration: Clinical Trials.gov ID: NCT02547532). Written informed consent was obtained from each subject and sputum collection were performed according to the local ethical committee guidelines.
Sputum samples collection and processing

Spontaneous or induced sputum was obtained from all study subjects. Induced sputum was obtained after inhalation of hypertonic saline (4.5%) following international guidelines. Sputum obtained from each subject was processed by the use of dithiothreitol (1:1) to obtain a liquid sputum specimen. This liquid sputum was divided into 3 aliquots: 1) one aliquot was used for Colony Forming Units (CFU) quantitation; 2) a second aliquot was processed for cytospin and surrnant preparations. May-Grunwald-Giemsa stained cytospins were used for cell count and cellular type differentiation. In the cytospin preparations a minimum number of 200 cells (range: 200–300 cells for all cases) was counted for each subject. In all cases squamous epithelial cells number was <80% of total cells counted (squamous epithelial cells percentage for all patient groups, mean±SD: 41.23±11.9). Supernatants were used for ELISA analysis of secreted cytokines related to innate immunity response and to neutrophilic activation (CCL3, CCL5, CXCL8, IL-27, LTβ4, MPO, Table S1); 3) a third aliquot was used for bacterial RNA/DNA extraction (QiaGen, Pathogen Mini Kit, Cat. N. 54104) and qRT-PCR analysis. Bacterial analysis was performed using RT-PCR and Syber Green technology. Bacterial analysis included: total bacterial load (16S), Moraxella catarrhalis, Haemophilus influenzae, Streptococcus pneumoniae, and Staphylococcus aureus. Total bacterial load (16S) was quantified after sputum RNA/DNA extraction. DNA standard for qRT-PCR was prepared from pure DNA cultures of Escherichia coli to generate a standard curve. The standard curve was performed in triplicate with regression coefficients close to 1 (range of $R^2$ values: 0.984—0.994 for E. coli) and showed a linear increase within the range of DNA concentration utilized, as previously reported. Genesig standard kits (Genesig Standard kit handbook HB10.04.09, published date 13/11/2017), including positive and negative controls for each of the bacteria studied, were used for qRT-PCR analysis of P. aeruginosa (RegA), H. influenzae (OMP P6), M. catarrhalis (copB), S. pneumoniae (alpha-fucosidase gene), S. aureus (FEMB gene). Genesig’s Manufacturers’ instructions were carefully followed for bacterial RT-PCR quantification. RT-PCR cycling conditions were: 95 °C for 5 min (PCR initial activation step); 40 amplification cycles of 95 °C for 5 s (denaturation) and 60 °C for 10 s (combined annealing/extension), followed by melting curve analysis to ensure the specificity of the PCR amplification. For each reaction, negative controls were run in triplicate, consisting of primers, PCR Mastermix and sterile water instead of DNA template. Amplification, data acquisition, and cycle threshold (CT) values analysis was performed using the Rotor Gene Q software (Rotor-Gene Q Series Software 2.0.2). Data were expressed as copies/ml of collected sputum from each patient studied. Routine bacteriological methods for identification of bacteria were used. The detection limit for sputum cultures was $10^3$ colony forming units (CFU)xml$^{-1}$ of each sampled sputum. A comparative analysis between the traditional methodology (CFU) and the RT-PCR bacterial quantitation was also performed.

Quantitation of inflammatory cells in sputum samples

The total number of inflammatory and buccal epithelial cells was quantified in sputum samples (cells/ml) and cellular types were counted in each cytospin preparation. The percentage of neutrophils, eosinophils, macrophages, lymphocytes and squamous epithelial cells was calculated for each subject studied.

ELISA tests for measurement of mediators

Sputum supernatants were aliquoted and stored at −80 °C until their use for the ELISA assays that have been summarized in Table S1. ELISA assays were performed in sixteen patients per group (n=80) consecutively enrolled in this study. These assays were performed according to the manufacturers’ instructions.

Statistical analysis

Group data were expressed as mean ± standard deviation (SD) for functional data and median (range) or interquartile range (IQR) for morphologic data. Differences between groups were analyzed using analysis of variance (ANOVA) for functional data. The ANOVA test was followed by the unpaired t-test for comparison between groups. The Kruskal-Wallis test applied for morphologic data, when significant, was followed by the Mann-Whitney U test for comparison between groups. Correlation coefficients were calculated using the Spearman rank method. Probability values of $p<0.05$ were considered significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA, USA).
Results

Clinical characteristics of subjects

We obtained and studied sputum samples from 102 subjects: 20 with cigarette smoke-associated stable COPD, 21 AATD-noT, 20 AATD-AT, 20 CS and 21 CON (Table 1). Cigarette smoke-associated COPD patients were older than control subjects (both CS and CON), had a similar history of smoking to CS, and their spirometry values showed milder to severe bronchial obstruction. Compared to cigarette smoke-associated COPD patients, AATD-AT patients were younger, had a reduced history of smoking, similar spirometry and DLCO values as well as emphysema grading on high resolution computed tomography (HRCT) but increased total lung capacity (TLC) values. AATD-noT patients had intermediate characteristics between cigarette smoke-associated COPD and AATD-AT patients. They were of similar age to AATD-AT patients and thus younger than cigarette smoke-associated COPD. Their smoking history was similar to AATD-AT patients, but lower than cigarette smoke-associated COPD. They did not show marked airflow obstruction at spirometry and relatively low emphysema grading on HRCT of the chest, thus differing from both cigarette smoke-associated COPD and AATD-AT patients. They showed moderate levels of airflow obstruction at spirometry and slightly lower emphysema grading on HRCT of the chest compared to AATD-AT patients.

All AATD patients had AAT plasma levels below the normal range. In AATD-AT, circulating AAT levels, measured just before augmentation therapy treatment, were lower compared to AATD-noT patients (28.03±18 vs 42.18±25 mg/dl; p=0.0545).

Genotype of AATD patients

The genotypes of AATD-noT patients were: PI*ZZ 8 cases, PI*SZ 6, PI*ZM_wurzburg 1, PI*ZM_varallo 1, PI*ZQ_brescia 1, PI*ZQ_parma 1, PI*SS 1, PI*Z new variant 1, PI*S new variant 1; the AAT genotypes of AATD-AT were: PI*ZZ 13, PI*SZ 2, PI*ZY_orzinuovi 1, PI*SP_lowel 1, PI*Q0_granitefalls Q0_granitefalls 1, PI*Q0_brescia Q0_brescia 1, PI*ZQ0_parma 1.

Inflammatory cells in the sputum

The results of the histochemical analysis are summarized in Table 2 and Figure 1A–E. Total sputum inflammatory cells did not differ significantly across the five groups (Kruskal-Wallis test, p=0.6777). Differential cell counts reported as percentage, however, were different between the study groups. Neutrophils percentage (Kruskal-Wallis: 0.0137) was increased in cigarette smoke-associated COPD patients compared to CON and AATD-AT groups (Mann-Whitney: p=0.0015 and p=0.0135, respectively), and was also different from the AATD-noT group (p=0.0446). The percentage of eosinophils (Kruskal-Wallis: p=0.0508) tended to be higher in cigarette smoke-associated COPD, AATD-noT and AATD-AT compared to CON (Mann-Whitney: p=0.0229, p=0.0345 and p=0.0098, respectively). The percentage of lymphocytes (Kruskal-Wallis: p=0.0955) showed a higher trend in CS, cigarette smoke-associated COPD, and AATD-noT compared to CON (Mann-Whitney: p=0.0366, p=0.0141 and p=0.0439, respectively).

The percentage of macrophages was not statistically different across the five groups at the Kruskal-Wallis test (p=0.0560), even though a higher trend was observed in CS and AATD-noT compared to CON (Mann-Whitney: p=0.0050 and p=0.0203, respectively) (Table 2).

Inflammatory mediators measured by ELISA in the sputum supernatants

ELISA data are reported in Table 3 and Figure 2. Table S1 shows the ELISA tests used. CCL3 (MIP1α), CCL5 (RANTES), LTB4 and MPO levels did not differ across the five patient groups studied (Figure 2A–D). No significant differences were found in CXCL8 level between the five groups (Kruskal-Wallis: p=0.0881) studied (Figure 2E). Sputum IL-27 levels in cigarette smoke-associated COPD were similar to that in AAT-noT, but significantly increased compared to AATD-AT and CON (p=0.0297 and p=0.0136, respectively).

ELISA tests considerations

Considering patients and control subjects studied by ELISA tests (n=80, 16 cases for each group), all the significant changes reported when all cases have been studied for quantitation (n=102, 20–21 cases for each group) of functional data (Table 1), cell count (Table 2) and bacterial load (Figures 3 and 4) have been maintained. The percentage of neutrophils was as well significantly reduced in AATD-AT (n=16) patients compared to cigarette smoke-associated COPD (n=16); M. catarrhalis (copies/ml) and S. pneumoniae (copies/ml) were as well reduced in AATD-AT (n=16) patients compared to cigarette smoke-associated COPD (n=16) (p=0.050 and p=0.002, respectively).

Table 1

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Table 2

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</table>

Figure 1A (copies/ml) were as well reduced in AATD-AT (n=16) α and MPO levels did not differ across the groups (Kruskal-Wallis test, p=0.0560). The percentage of eosinophils (Kruskal-Wallis: p=0.0508) tended to be higher in cigarette smoke-associated COPD, AATD-noT and AATD-AT compared to CON (Mann-Whitney: p=0.0229, p=0.0345 and p=0.0098, respectively). The percentage of lymphocytes (Kruskal-Wallis: p=0.0955) showed a higher trend in CS, cigarette smoke-associated COPD, and AATD-noT compared to CON (Mann-Whitney: p=0.0366, p=0.0141 and p=0.0439, respectively).

The percentage of macrophages was not statistically different across the five groups at the Kruskal-Wallis test (p=0.0560), even though a higher trend was observed in CS and AATD-noT compared to CON (Mann-Whitney: p=0.0050 and p=0.0203, respectively) (Table 2).
Comparison of relevant inflammatory cells and biomarkers in patients with AATD-noT with bronchial obstruction and cigarette smoke-associated COPD

Since 42% of AATD patients not in the augmentation therapy (9 out of 21 AATD-noT) had a similar bronchial obstruction when compared to cigarette smoke-associated COPD patients, we compared them for the relevant inflammatory markers studied. The number of neutrophils (cells/ml and percentage), IL-27 and CCL5 proteins were not significantly different in the two groups studied (Mann-Whitney: \( p=0.186 \), \( p=0.194 \), \( p=0.053 \) and \( p=0.192 \), respectively). A slight but significant increase of \( M. \) catarrhalis (copies/ml) (\( p=0.016 \)) and of \( S. \) pneumoniae (copies/ml) (\( p=0.0125 \)) was observed in cigarette smoke-associated COPD compared to AATD-noT patients with bronchial obstruction (\( n=9 \)). The reasons for this difference need a more extended analysis involving a higher number of AATD-noT patients.

Colony-forming units (CFU) in the sputum

In the group of CON, 20 subjects showed the presence of normal bacterial flora, and one showed growth of \( S. \) aureus. In the group of CS, 16 subjects showed normal bacterial flora, 2 showed \( H. \) parainfluenzae, and 2 showed \( H. \) influenzae. In cigarette smoke-associated COPD, 11 patients showed normal bacterial flora, 1 patient \( H. \) influenzae, 2 patients \( S. \) pneumoniae, 3 patients \( S. \) aureus, 1 patient \( S. \) aureus plus \( P. \) aeruginosa, 1 patient \( S. \) aureus plus \( H. \) influenzae, and 1 patient \( S. \) pneumoniae plus \( H. \) influenzae. In AATD-noT, 16 patients had normal bacterial flora, 2 patients \( P. \) aeruginosa, 1 patient \( S. \) pneumoniae, 1 patient \( S. \) aureus, and 1 patient \( S. \) aureus plus \( H. \) influenzae. In AATD-AT, 15 patients had normal bacterial flora, 1 patient \( P. \) aeruginosa, 1 patient \( H. \) influenzae, 1 patient \( S. \) aureus, 1 patient \( S. \) aureus plus \( H. \) influenzae, and 1 patient \( H. \) influenzae plus \( M. \) catarrhalis.
Quantification of bacterial load in the sputum

When data were expressed as absolute numbers (Figure 3) (copies/ml), AATD-NoT and AATD-AT patients had significantly lower total bacterial load (16S) than cigarette smoke-associated COPD ($p=0.0081$ and $p=0.0223$) (Figure 3A). AATD-noT and AATD-AT had a lower number of copies/ml of *Moraxella catarrhalis* than cigarette smoke-associated COPD ($p=0.0115$ and $p=0.0127$) (Figure 3B). Cigarette smoke-associated COPD patients and CS had a higher number of copies/ml compared to CON ($p=0.0009$ and $p=0.0259$). AATD-noT and AATD-AT also had significantly fewer copies/ml of *Streptococcus pneumoniae* compared to cigarette smoke-associated COPD ($p=0.0013$ and $p=0.0001$). CS also differed from AATD-AT ($p=0.0499$) (Figure 3C). No significant differences were observed for *Pseudomonas aeruginosa* (Kruskal-Wallis: $p=0.0789$), *Haemophilus influenzae* (Kruskal-Wallis: $p=0.1666$) and *Staphylococcus aureus* (Kruskal-Wallis: $p=0.2246$) across the five groups studied (Figure 3D–F).

To evaluate changes in the relative proportions of each bacterium studied, we expressed these data as a percentage of the total load value constituted by the sum of the five bacteria studied (Figure 4). In cigarette smoke-associated COPD, the percentage of *M. catarrhalis* was higher compared to CON, AATD-noT and AATD-AT ($p=0.0015$, $p=0.0063$ and $p=0.0055$, respectively). Again, in cigarette smoke-associated COPD, the percentage of *S. pneumoniae* was higher compared to CON, CS, AATD-noT and AATD-AT ($p=0.0006$, $p=0.0094$, $p=0.0008$ and $p<0.0001$, respectively) (Figure 4). The percentages of *P. aeruginosa*, *H. influenzae* and *S. aureus* did not significantly differ across the five groups (Kruskal-Wallis: $p=0.2720$, $p=0.1598$ and $p=0.1358$, respectively) (Figure 4).

Correlations between clinical parameters, number of inflammatory cells, signaling mediators expression and bacterial load in the sputum

When we grouped patients with cigarette smoke-associated COPD (n=20), CS (n=20) and CON (n=21), the percentage of neutrophils in the sputum was inversely correlated with FEV$_1$% predicted values ($R=-0.301$, $p=0.0218$) and positively correlated with *M. catarrhalis*.
Figure 1 Total inflammatory cells (cells/ml) (A), and percentages of neutrophils (B), eosinophils (C), lymphocytes (D) and macrophages (E) in cytospin preparations from control non-smokers (CON, n=21), control smokers (CS, n=20), cigarette smoke-associated COPD (n=20), α-anti trypsin deficiency not in therapy (AATD-noT, n=21) and AATD patients on augmentation therapy (AATD-AT, n=20). The total number of inflammatory cells, and the percentage of eosinophils, macrophages and lymphocytes did not differ (K. Wallis: p=0.6777, p=0.0508 and p=0.0955, respectively) in the five groups studied. The percentage of neutrophils (K. Wallis: p=0.0137) was significantly higher in cigarette smoke-associated COPD compared to CON (p=0.0015) and AATD-AT (p=0.0135) (B). Cigarette smoke-associated COPD also differed from AATD-NoT (p=0.0446). Data are presented as median and interquartile range. Statistical analysis between groups: Mann-Whitney U test, reported only when the Kruskal-Wallis (KW) test resulted significant.

Abbreviations: CNS, control non-smokers; CS, control smokers; COPD, chronic obstructive pulmonary disease; α1ATD-A.T., α-anti trypsin deficiency patients not in therapy; α1ATD-noT, α-anti trypsin deficiency patients on augmentation therapy.

(copies/ml) (R=0.27, p=0.0344), S. pneumoniae (copies/ml) (R=0.367, p=0.0045) and P. aeruginosa (copies/ml) (R=0.319, p=0.0133). The percentage of neutrophils (n=48) also correlated with CXCL8 (pg/ml) in the supernatants (R=0.395, p=0.0068) and MPO (ng/ml) (R=0.436, p=0.0047). However, multiple regression analysis considering neutrophils (%) as dependent variable and the six quantified biomarkers (CCL3, CCL5, CXCL8, IL-27, LTB4, MPO) as independent variables showed a significant positive association with MPO (ng/ml) (R=0.587, p=0.0007). When we grouped patients with cigarette smoke-associated COPD (n=20 or 16 for ELISA tests) and AATD-AT (n=20 or 16 for ELISA tests), M. catarrhalis (copies/ml) and S. pneumoniae (copies/ml) significantly and positively correlated with the percentage of neutrophils (R=0.489, p=0.0050 and R=0.570, p=0.0011, respectively) and IL-27 protein (pg/ml) (R=0.377, p=0.0423 and R=0.432, p=0.0199, respectively). Scored emphysema in all patients with AATD (n=41) correlated significantly with FEV₁% (R=−0.68, p<0.0001), FEV₁/FVC% (R=−0.58, p=0.0019), lung diffusion capacity of carbon monoxide (DLCO)% (R=−0.74, p=0.0006) and with residual volume (RV)% (R=0.47, p=0.022).

Spontaneous versus induced sputum analysis and day-to-day variability
In all patients and control subjects studied (n=102) spontaneous sputum was obtained from sixty patients and 42 patients underwent to the sputum induction procedure. We did not find significant differences between spontaneous and induced sputum samples for neutrophil % (Mann-Whitney: p=0.391), IL-27 protein (p=0.777), CCL5 protein (p=0.569), S. pneumoniae (copies/ml and percentage) (p=0.645 and p=0.485 respectively) and M. catarrhalis (copies/ml and percentage) (p=0.650 and 0.424 respectively). The absolute number of neutrophils (cells/ml) was slightly increased in spontaneous sputum compared
Table 3  Inflammatory mediators and cytokines in sputum supernatants samples of cigarette smoke-associate chronic obstructive pulmonary disease (COPD), alpha-1 anti-trypsin deficiency patients and control subjects

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<th>Control Smokers</th>
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<td></td>
</tr>
<tr>
<td>M/F</td>
<td>11/5</td>
<td>9/7</td>
<td>13/3</td>
<td>11/5</td>
<td>11/5</td>
<td></td>
</tr>
<tr>
<td>CCL3 (pg/ml)</td>
<td>149 (31–1242)</td>
<td>181 (73–1494)</td>
<td>199 (28–624)</td>
<td>253 (0–1710)</td>
<td>154 (14–801)</td>
<td>0.2192</td>
</tr>
<tr>
<td>CCL5 (pg/ml)</td>
<td>136 (31–739)</td>
<td>67 (18–531)</td>
<td>34 (0–1011)</td>
<td>82 (5–778)</td>
<td>96 (4–619)</td>
<td>0.2210</td>
</tr>
<tr>
<td>CXCL8 (pg/ml)</td>
<td>1298 (0–9492)</td>
<td>5809 (222–95870)</td>
<td>6250 (534–63940)</td>
<td>3665 (267–71480)</td>
<td>4741 (356–16700)</td>
<td>0.0881</td>
</tr>
<tr>
<td>IL-27 (pg/ml)</td>
<td>6 (0–545)</td>
<td>279 (0–610)*</td>
<td>113 (0–614)*</td>
<td>51 (0–614)</td>
<td>0 (0–608)*</td>
<td>0.0475</td>
</tr>
<tr>
<td>LTB4 (pg/ml)</td>
<td>2014 (107–5903)</td>
<td>1390 (97–20150)</td>
<td>1763 (217–6171)</td>
<td>1833 (514–10850)</td>
<td>1562 (104–9246)</td>
<td>0.7551</td>
</tr>
<tr>
<td>MPO (ng/ml)</td>
<td>1043 (12–1491)</td>
<td>1280 (0–1496)</td>
<td>1469 (130–1483)</td>
<td>1448 (174–1500)</td>
<td>1429 (424–1485)</td>
<td>0.0966</td>
</tr>
</tbody>
</table>

Note: Data are presented as median (range). Mann-Whitney U test, applied only when the Kruskal-Wallis test resulted significant. *p<0.05 from COPD patients; **p<0.05 from control non-smokers. Bold value indicates statistically significant variation.

Abbreviations: AATD-noT, alpha-1 antitrypsin deficiency patients not in therapy; AATD-AT, alpha-1 antitrypsin deficiency patients in augmentation therapy; M, male; F, female; CCL3, chemokine (CC motif) ligand 3; CCL5, chemokine (CC motif) ligand 5; IL, interleukin; LTB4, leukotriene-B4; MPO, myeloperoxidase.

Discussion

The main finding of our study is the inflammatory and microbiological profiles in the lower airways of patients with AATD differ from those observed in cigarette smoke-associate COPD. Compared with cigarette smoke-associated COPD patients, patients with AATD, whether on AT or not, had lower amount of neutrophils in sputum and the atrophic load of bacteria (M. catarrhalis and S. pneumoniae, in particular). Cigarette smoking per se affected the microbiota composition. Moreover, AATD patients, but not AAT-noT patients, had decreased IL-27 levels in sputum compared with cigarette smoke-associated COPD. The observed differences in inflammatory profile among cigarette smoke-associated COPD and AAT-AT patients are noteworthy as the two groups have a similar clinical and functional profile.

The inclusion criteria for AATD patients were functional parameters similar to induced one. Spontaneous sputum was obtained from 15 cigarette smoke-associated COPD, 15 AAT-AT, 13 AAT-noT, 9 AATD-AT patients. The study by Stone’s and colleagues suggest to optimize the analysis using 2 consecutive sputum samples collected in different days. Moreover, data obtained were considered significant only when multiple and paired statistical tests were significant. We believe that the higher number of patients used in each group (20–21), the statistical approach used, one collection per patient or control subject using 20–21 subjects used for all the statistical tests adopted and the similar technical approach performed our sputum analysis using a single sample from each studied patient or control subject.

Day-to-day variability has been found for neutrophil and some cytokine quantitations in sputum of COPD and AATD patients. The study by Stone’s and colleagues suggest to optimize the analysis using 2 consecutive samples. Moreover, data obtained were considered significant only when multiple and paired statistical tests were significant. We believe that the higher number of patients used in each group (20–21), the statistical approach used, one collection per patient or control subject using 20–21 subjects used for all the statistical tests adopted and the similar technical approach performed our sputum analysis using a single sample from each studied patient or control subject.

AATD-AT patients, but not AATD-noT patients, had increased IL-27 levels in sputum compared with cigarette smoke-associated COPD patients, patients with AATD, whether on AT or not, had lower amount of neutrophils in sputum and the atrophic load of bacteria (M. catarrhalis and S. pneumoniae, in particular). Cigarette smoking per se affected the microbiota composition. Moreover, AATD patients, but not AAT-noT patients, had decreased IL-27 levels in sputum compared with cigarette smoke-associated COPD. The observed differences in inflammatory profile among cigarette smoke-associated COPD and AAT-AT patients are noteworthy as the two groups have a similar clinical and functional profile.

Table 3  Inflammatory mediators and cytokines in sputum supernatants samples of cigarette smoke-associate chronic obstructive pulmonary disease (COPD), alpha-1 anti-trypsin deficiency patients and control subjects

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Control non-Smokers</th>
<th>Control Smokers</th>
<th>Cigarette smoke-associated COPD</th>
<th>AATD-noT</th>
<th>AATD-AT</th>
<th>Kruskal-Wallis (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>11/5</td>
<td>9/7</td>
<td>13/3</td>
<td>11/5</td>
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Note: Data are presented as median (range). Mann-Whitney U test, applied only when the Kruskal-Wallis test resulted significant. *p<0.05 from COPD patients; **p<0.05 from control non-smokers. Bold value indicates statistically significant variation.

Abbreviations: AATD-noT, alpha-1 antitrypsin deficiency patients not in therapy; AATD-AT, alpha-1 antitrypsin deficiency patients in augmentation therapy; M, male; F, female; CCL3, chemokine (CC motif) ligand 3; CCL5, chemokine (CC motif) ligand 5; IL, interleukin; LTB4, leukotriene-B4; MPO, myeloperoxidase.
characteristics of the two AATD groups indicate that they correspond to different time-points in the natural development of AATD-related lung disease, passing from the less severe airway and parenchymal inflammatory involvement in AAT-noT patients to severe COPD in AAT-AT patients.

Our data confirm an increased sputum neutrophilia in cigarette smoke-associated COPD in agreement with many other published reports whilst the decreased sputum neutrophil percentage and a trend to decline in absolute numbers in AATD-AT patients compared to cigarette smoke-associated COPD also confirm previous observations. However, the mechanisms causing a reduction in neutrophil accumulation in the lower airways and their decreased activation after augmentation therapy are controversial. Neutrophil elastase stimulates epithelial cells of the lower airways to release CXCL8 and increased BAL levels of CXCL8 were reported in AATD patients compared to cigarette smoke-associated COPD; moreover, colonized AATD or colonized cigarette smoke-associated COPD patients have increased sputum CXCL8 concentrations compared to the same non-colonized group of patients. However, we did not find markedly significant differences in sputum CXCL8 levels between AATD-AT patients and cigarette smoke-associated COPD patients with a similar degree of airflow obstruction, suggesting that augmentation therapy reduces sputum neutrophilia in these patients, thus acting with other molecular mechanisms.

In the present paper, as an original contribution, AATD-AT patients compared with cigarette smoke-associated COPD have a significant reduction in sputum IL-27 protein levels, suggesting a role for this cytokine in reducing sputum neutrophilia in these patients. Interestingly, the sputum levels of IL-27 protein were also positively correlated with the specific bacterial load of *M. catarrhalis*.

**Figure 2** Chemokines and cytokines quantified (pg/ml) in the sputum supernatants of control non-smokers (CON, n=16), control smokers (CS, n=16), cigarette smoke-associated chronic obstructive pulmonary disease (COPD, n=16), α1-anti trypsin deficiency not in therapy (AATD-noT, n=16) and AATD in augmentation therapy (AATD-AT, n=16). Data are expressed as median and interquartile range. The Kruskal-Wallis (KW) test was used for multiple comparisons followed by the Mann-Whitney U-test for comparison between groups. CCL3, CCL5, LTB4 and MPO levels were similar in all five patient groups (A-D). CXCL8 (IL-8) was increased in CS, cigarette smoke-associated COPD and AATD-AT in comparison to CON, even though the difference did not reach statistical significance at the Kruskal Wallis test (E). IL-27 was significantly higher in cigarette smoke-associated COPD and in CS compared to CON. Cigarette smoke-associated COPD also showed higher values compared to AATD-AT (F).

**Abbreviations:** CCL3, chemokine (CC motif) ligand 3; CCL5, chemokine (CC motif) ligand 5; IL, interleukin; LTB4, leukotriene-B4; MPO, myeloperoxidase.
and S. pneumoniae (copies/ml) in AATD-AT patients. In addition, when all AATD patients were grouped together, sputum IL-27 levels correlated positively with FEV1% predicted values, suggesting a role for the augmentation therapy in reducing sputum IL-27.

Monocytes/macrophages and neutrophils are the major source of IL-27 production during sepsis and blocking of IL-27 production in neutrophils in vitro reduces bacterial survival. Modulation of neutrophil function by IL-27 is also reported in intracerebral hemorrhage. Interestingly, IL-27 pre-treatment of neutrophils and monocytes in vitro decreases CXCL8 production and in a model of zymosan-induced peritonitis, the administration of IL-27 reduces neutrophil recruitment to the peritoneal cavity.

We also observed a concomitant reduction of sputum neutrophil numbers and IL-27 protein levels in AATD-AT patients compared to cigarette smoke-associated COPD, with both study groups having a similar degree of airflow obstruction. For the above reasons we speculate that the reduction in sputum neutrophil numbers may be influenced, at least in part, by IL-27 activity. However, we cannot exclude that the concomitant decline in both sputum neutrophil and IL-27 levels could also be an effect of the neutrophil decrement per se since neutrophils synthesize IL-27 protein.

CC-chemokines, in particular CCL3 and CCL5, have been reported to be involved in the pathogenesis of cigarette smoke-associated COPD and in the induction of neutrophil migration and activation. In the current study, at variance with our previous study performed on bronchial biopsies, we did not observe any significant change in sputum CCL3 and CCL5 levels among the five groups of subjects studied. Differences in the release of inflammatory mediators in different compartments of the lower airways have already been observed in many different chronic inflammatory diseases. Furthermore, we observed no
significant difference in sputum MPO and LTB4 levels between AATD-AT patients (treated for at least 3 months), in comparison with cigarette smoke-associated COPD with a similar degree of obstruction (Figure 2C and D).

When comparing sputum MPO in cigarette smoke-associated COPD and AATD-noT patients with a similar degree of airflow obstruction there are conflicting results in the literature (Table 4). Whereas most of the previous studies in the same group of patients have shown a significant increase of sputum leukotriene B4 (Table 4). These differences may be explained by the lack of untreated AATD patients with severe/very severe COPD in our study, the differences in the ELISA assays used for measuring these mediators and the length of augmentation therapy treatment in the different studies (Table 4).

There are few published studies that have investigated the total and specific bacterial load and their correlations with sputum inflammation in patients with AATD-AT and AATD-noT. Both cigarette smoke-associated COPD and AATD patients in stable phase have increased sputum CXCL8 and LTB4 levels when colonized compared to non-colonized patients, suggesting a role for bacteria in increasing neutrophil-related inflammation. In our study, only a minority of patients showed sputum CFU for different bacterial species making impossible to directly compare inflammatory cells and mediators between colonized and non-colonized patients. Interestingly, we report here for the first time the absolute (expressed as copies/ml) and percentage values of the total and specific bacterial load in the five groups of subjects investigated.

We show here that cigarette smoking per se had a clear effect on microbiota composition, since CS were similar to CON for total bacterial load (16S) but showed significantly increased M catarrhalis and S. pneumoniae when compared to CON which is in keeping with previous studies. The highest values of total bacterial load were observed in cigarette smoke-associated COPD. P. aeruginosa showed a trend to be increased in cigarette smoke-associated COPD, AATD-noT and AATD-AT with respect to CON confirming earlier studies using different methods. M. catarrhalis and S. pneumoniae copies/ml were increased in cigarette smoke-associated COPD compared to CON and significantly decreased in AATD-AT, having a similar degree of airflow obstruction.
### Table 4: Previous studies on the quantification of LTB4 and MPO in sputum samples of patients with AATD/COPD compared to cigarette smoke-associated COPD

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Augmentation T.</th>
<th>Compartments</th>
<th>LTB4</th>
<th>MPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill et al. 2000</td>
<td>42 COPD (mod/sev) vs 39 PiZ α1ATD (sev)</td>
<td>No</td>
<td>Sputum</td>
<td><img src="https://via.placeholder.com/15" alt="↑" /></td>
<td><img src="https://via.placeholder.com/15" alt="↑" /></td>
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<tr>
<td>Stockley et al. 2002</td>
<td>12 AATD/COPD, longitudinal, one month</td>
<td>Yes, no vs one month</td>
<td>Sputum</td>
<td><img src="https://via.placeholder.com/15" alt="↓" /></td>
<td><img src="https://via.placeholder.com/15" alt="→" /></td>
</tr>
<tr>
<td>Gompertz et al. 2006</td>
<td>22 COPD (mod/sev) vs 14 PiZ α1ATD (mod/sev)</td>
<td>No</td>
<td>Sputum</td>
<td><img src="https://via.placeholder.com/15" alt="↑" /></td>
<td><img src="https://via.placeholder.com/15" alt="↑" /></td>
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<tr>
<td>Griese et al. 2007</td>
<td>52 cystic fibrosis, longitudinal, one month</td>
<td>Inhaled AAT, one month</td>
<td>Induced Sputum</td>
<td><img src="https://via.placeholder.com/15" alt="↓" /></td>
<td><img src="https://via.placeholder.com/15" alt="→" /></td>
</tr>
<tr>
<td>Stone et al. 2012</td>
<td>12 COPD (sev) vs 12 A1ATD (sev)</td>
<td>No</td>
<td>Sputum</td>
<td><img src="https://via.placeholder.com/15" alt="↑" /></td>
<td><img src="https://via.placeholder.com/15" alt="↓" /></td>
</tr>
</tbody>
</table>

**Note:** This table does not include the human studies performed in serum/plasma, bronchoalveolar lavage and “in vitro” studies from primary cells of COPD patients isolated from blood and/or lower airways.

**Abbreviations:** mod, moderate; sev, severe; augmentation T, augmentation therapy; arrows indicate significant changes observed in the AATD with COPD group compared to cigarette smoke-associated COPD.
This decrement in absolute numbers in AATD-AT was also associated to a decreased relative proportion of *M. catarrhalis* and *S. pneumoniae* when compared to cigarette smoke-associated COPD. Our observation of a positive correlation of *M. catarrhalis* and *S. pneumoniae* bacterial load with neutrophil percentage and IL-27 protein in the sputum of cigarette smoke-associated COPD and AATD-AT patients suggests a possible inter-relationship between specific bacterial load, IL-27 secretion and neutrophil recruitment. This is in keeping with recent observations that IL-27 impairs host innate immunity against pneumococcal infection.\(^{39}\) The mechanistic inter-relationship between neutrophil recruitment, IL-27 production and bacterial load, may be worth to be investigated in large controlled clinical trials of augmentation therapy in AATD.

In conclusion, compared to cigarette smoke-associated COPD, AATD-AT (COPD) patients have a distinct airway inflammatory and microbiological profile. The decreased sputum bacterial load and IL-27 levels in AATD-AT patients suggests that augmentation therapy play a role in these changes. The mechanistic inter-relationship between neutrophil recruitment, IL-27 production and bacterial load, may be worth to be investigated in large controlled clinical trials of augmentation therapy in AATD.

**Data sharing statement**

The authors intend to share individual deidentified participant data, including demographics, detailed clinical information and cell and supernatant measurements in sputum. The data will be accessible by emailing the authors after the manuscript is published. Subject’s identity information will not be available for privacy reasons.

**Acknowledgments**

This work was supported by grants from Grifols Inc. and from the Istituti Clinici Scientifici Maugeri, IRCCS and by Grifols Inc. Grifols Inc. with a two-year grant. Grifols funding included laboratory supplies, travel expenses for collecting samples around the Country and a fellowship for a Ph.D. The authors report no other conflicts of interest in this work.

**Author contributions**

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

**Disclosure**

This study was funded by Istituti Clinici Scientifici Maugeri, IRCCS and by Grifols Inc. Grifols Inc. with a two-year grant. Grifols funding included laboratory supplies, travel expenses for collecting samples around the Country and a fellowship for a Ph.D. The authors report no other conflicts of interest in this work.

**References**

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**Lung function tests and volumes**

Pulmonary function tests were performed as previously described according to guideline recommendations. Pulmonary function tests included measurements of FEV₁ and FEV₁/FVC under baseline conditions in all subjects (6200 Autobox Pulmonary Function Laboratory; Sensormedics Corp., Yorba Linda, CA, USA). To assess the reversibility of airflow obstruction and post bronchodilator functional values, the FEV₁ and FEV₁/FVC% measurements in the groups of subjects with FEV₁/FVC% ≤70% pre-bronchodilator was repeated 20 min after the inhalation of 0.4 mg of salbutamol.

**Table S1** Summary of the ELISA assays performed on sputum supernatants

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Manufacturer website</th>
<th>Catalogue code</th>
<th>Standard curve sensitivity range</th>
<th>Sputum samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3</td>
<td>Abnova <a href="http://www.abnova.com">www.abnova.com</a></td>
<td>KA0388</td>
<td>15.6–1000 pg/mL</td>
<td>Not diluted</td>
</tr>
<tr>
<td>CCL5</td>
<td>Abnova <a href="http://www.abnova.com">www.abnova.com</a></td>
<td>KA0411</td>
<td>15.6–1000 pg/mL</td>
<td>Not diluted</td>
</tr>
<tr>
<td>CXCL8</td>
<td>R&amp;D Systems <a href="http://www.bio-techne.com">www.bio-techne.com</a></td>
<td>D8000C</td>
<td>31.3–2000 pg/mL</td>
<td>Diluted 1:100</td>
</tr>
<tr>
<td>IL-27</td>
<td>Cloudclone <a href="http://www.cloud-clone.com">www.cloud-clone.com</a></td>
<td>SEA385Hu</td>
<td>15.6–1000 pg/mL</td>
<td>Not diluted</td>
</tr>
<tr>
<td>LTB4</td>
<td>R&amp;D Systems <a href="http://www.bio-techne.com">www.bio-techne.com</a></td>
<td>KGE006B</td>
<td>3.7–2500 pg/ml</td>
<td>Diluted 1:10</td>
</tr>
<tr>
<td>MPO</td>
<td>R&amp;D Systems <a href="http://www.bio-techne.com">www.bio-techne.com</a></td>
<td>DMYE008B</td>
<td>0.014–10 ng/ml</td>
<td>Diluted 1:100</td>
</tr>
</tbody>
</table>

**Abbreviations:** CCL3, chemokine (CC motif) ligand 3; CCL5, chemokine (CC motif) ligand 5; IL-27, interleukin-27; LTB4, leukotriene-B4; MPO, myeloperoxidase.

**Reference**