Neutrophilic inflammation and IL-8 levels in induced sputum of alpha-1-antitrypsin PiMZ subjects

M Malerba, F Ricciardolo, A Radaeli, C Torregiani, L Ceriani, E Mori, M Bontempelli, C Tantucci, V Grassi

Background: Severe alpha-1-antitrypsin deficiency (AATD), due to homozygosity for the protease inhibitor (Pi) Z allele, is a genetic risk factor for chronic obstructive pulmonary disease (COPD). In a previous study the sputum of severe AATD subjects with airflow obstruction showed a pattern of cellular inflammation similar to COPD patients. It is uncertain whether heterozygotes for the Z allele or intermediate deficiency (PiMZ) have an increased risk of developing COPD.

Methods: Sputum cell counts and the supernatant level of the neutrophil chemoattractant interleukin (IL)-8 were investigated by sputum induction in 10 non-smoker asymptomatic PiMZ subjects with normal pulmonary function, 10 patients with stable COPD, and 10 age matched normal subjects. Data are expressed as mean (SD).

Results: The mean (SD) number of neutrophils was significantly higher (p = 0.01) in the sputum of PiMZ subjects (84.5 (22.2) x10⁴/ml) and patients with COPD (126.9 (18.8) x10⁴/ ml) than in matched normal subjects (55.0 (8.7) x10⁴/ ml). IL-8 levels were increased in PiMZ subjects (828.5 (490.6) ng/ml; range 1003.0 ng/ml; range 1260–100 ng/ml) and in COPD patients (882.5 (524.3) ng/ml; median 934.9 ng/ml; range 1506–258 mg/ml) compared with normal subjects (3.5 (0.5) ng/ml; median 3.5 ng/ml; range 4.5–2.5 ng/ml). There was a significant positive correlation between IL-8 supernatant concentration and neutrophil count in PiMZ subjects (p = 0.036; r = 0.66). An inverse correlation was observed between the percentage of neutrophils and forced expiratory volume in 1 second (% predicted) in patients with COPD (p = 0.04; r = −0.43).

Conclusions: These findings indicate that PiMZ subjects without airflow obstruction may have an IL-8 related neutrophilic inflammation in the airways, similar to stable COPD patients, suggesting an increased risk of developing pulmonary changes.

RESULTS

Ten consecutive non-smoking asymptomatic subjects (5 men and 5 women) of mean (SD) age 47.8 (9.7) years heterozygotes for AATD (PiMZ) (mean (SD) AAT blood level 108 (31.7) mg/dl) included in the Italian Registry and attending our Regional Reference Center of Brescia (Department of Internal Medicine) were studied in an ambulatory setting. Diagnosis of AAT deficiency was confirmed by phenotyping. At the time of inclusion all the subjects were in a stable condition and had been free from respiratory exacerbations for at least 4 weeks. A complete medical history was obtained for each subject. No subject had present or past history of exposure to any occupational factor and of passive smoking. All the subjects studied had normal lung function. Before the study other concomitant pulmonary diseases (such as bronchiectasis) were ruled out in PiMZ subjects by chest radiography and high resolution CT scans.

Abbreviations: AAT, alpha-1-trypsin; AATD, alpha-1-trypsin deficiency; COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; IL, interleukin; TLCO, carbon monoxide transfer factor
COPD
Ten patients with stable COPD (6 men, 4 women) of mean (SD) age 65.7 (13.3) years with PiMM (mean (SD) AAT blood level 140.3 (10.2) mg/dl) were enrolled in the study. The diagnosis of COPD was made according to the following standard criteria:
- age >40 years, current or previous history of smoking (>10 pack-years), history of chronic symptoms (cough with sputum production for at least 3 months in at least two consecutive years), and the presence of airflow obstruction (forced expiratory volume in one second/forced vital capacity (FEV₁/FVC <70%). At the time of the study all the patients were in a stable condition and had been free from acute exacerbations for at least 4 weeks. No patient was on systemic corticosteroids or had received antibiotics within the month preceding the study. Treatment with inhaled corticosteroids was withdrawn at least 4 weeks before entering the study.

Controls
The control group consisted of 10 healthy subjects, age matched to PiMZ subjects, (4 men, 6 women) of mean (SD) age 46.1 (21.7) years known to be PiMM (mean (SD) AAT blood level 162.5 (18.5) mg/dl) with checked normal pulmonary function tests. They were excluded if they had a history of respiratory disease or experienced atopy.

The study protocol was approved by the local ethics board and all subjects gave their written informed consent to participate in the study which was conducted in accordance with the Helsinki Declaration.

Measurements
AAT assay and phenotyping
AAT serum concentrations were measured in all subjects by the nephelometry method (normal range 90–200 mg/dl). AAT phenotyping was performed by isoelectric focusing.

Pulmonary function tests
Lung function was measured in accordance with the American Thoracic Society (ATS) standard procedure.

Sputum induction procedure
After baseline FEV₁ and FVC measurements, subjects were pretreated with inhaled salbutamol (200 μg by metered dose inhaler) and 10 minutes later inhaled hypertonic (4%) sterile saline nebulised solution was given for three periods of 5 minutes at most by an ultrasonic nebuliser (DeVilbiss Ultranech 2000). The subjects were instructed to cough sputum into containers. If any symptoms occurred, nebulisation was discontinued.

Sputum processing
The collected sputum samples were examined within 2 hours. Sputum plugs originating from the lower respiratory tract were separated from the collected samples and then weighed. Dithiotreitol (DTT; Sputolysin; Calbiochem Corporation, San Diego, CA, USA) was freshly prepared in a 1:10 dilution with distilled water and added to the plugs in a volume (in μl) equal to four times the weight of the selected plugs (in mg). The test tube containing DTT and sputum was homogenised, left in a thermostat at 37°C for 20 minutes, and then diluted with a volume of phosphate buffered saline (PBS) equal to the DTT. The suspension was filtered through a 48 μm gauze and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and then frozen at -70°C for later analysis. The cell pellet was resuspended in PBS in a volume equal to the volume of DTT added at the beginning of the test, spun in a cytocentrifuge (PK120R ALC International), and stained with Diff-Quik. Cells were counted with a Burker's chamber haemocytometer; only samples with a cell viability of ≥50% and <20% squamous cell contamination were considered adequate. The differential cell count was expressed as the percentage of total non-squamous cells (%NSC) and as the number of cells × 10⁶/ml.

Sputum biochemistry
IL-8 was measured in sputum supernatant by the enzyme linked immunosorbent assay method using commercially available kits (Bender Med-Systems). This assay has been shown to function normally in the presence of DTT.

Statistical analysis
Statistical analysis was performed with the SPSS software package (SPSS; Chicago, IL, USA). A p value of <0.05 was considered significant. All values are expressed as mean (SD). Comparison of groups was performed by analysis of variance testing (ANOVA). Unpaired t tests were used when appropriate. Categorical data were compared using the Fisher exact test, and the Kruskal-Wallis test or Mann-Whitney test was used for unpaired data (when appropriate).

RESULTS
Clinical findings
The demographic and pulmonary functional characteristics of the patients studied are shown in table 1. The patients with COPD were significantly older (p = 0.019) whereas PiMZ subjects and controls were age matched. Plasma AAT levels were lower in PiMZ subjects than in the other groups (p = 0.002; v COPD p = 0.046; v controls p = 0.002). Patients with COPD had similar serum AAT levels to the healthy control subjects (p = 0.69).

The PiMZ and healthy control subjects did not have airflow obstruction, while COPD patients had significantly lower FEV₁ (% predicted) and FEV₁/FVC (%) than the other two groups (both p = 0.000). There were no significant differences in TLC values between the groups (p = 0.09; COPD v PiMZ p = 0.26; COPD v controls p = 0.12; table 1).

Sputum induction
The sputum induction results are shown in table 2. The mean percentage of total cells and epithelial cells did not differ significantly between the groups (p = 0.106 and p = 0.116). The mean (SD) neutrophil count was higher in PiMZ subjects (84.5 (22.2) × 10⁶/ml; 69.3 (18.2) %NSC) and in COPD patients (126.9 (18.8) × 10⁶/ml; 76.3 (9.9) %NSC) than in healthy control subjects (55.0 (8.7) × 10⁶/ml; 45.1 (7.2) %NSC; p = 0.000). In contrast, the mean (SD) number of macrophages was lower in PiMZ subjects (32.7 (11.3) × 10⁶/ml; 26.8 (9.4) %NSC) and in patients with COPD (30.1 (21.4) × 10⁶/ml; 18.5 (11.3) %NSC) than in healthy control subjects (61.6 (9.2) × 10⁶/ml; 50.5 (7.6) %NSC; p = 0.000). The lymphocyte population was similar in the three groups (p = 0.971). The PiMZ subjects and COPD patients showed an inverted macrophage/neutrophil ratio compared with normal subjects. The IL-8 levels in sputum supernatant of COPD patients (882.5 (524.3) ng/ml; median 934.9 ng/ml; range 1506–258 mg/ml) and PiMZ subjects (828.5 (490.6) ng/ml; median 1003.0 ng/ml; range 1260–100 ng/ml) were similar to (p = 0.88) but higher (p = 0.000) than IL-8 levels in healthy control subjects (3.5 (0.5) ng/ml; median 3.5 ng/ml; range 4.5–2.5 ng/ml).

A significant positive correlation was found between the supernatant concentration of IL-8 and neutrophil numbers in the induced sputum of PiMZ subjects (p = 0.036; r = 0.66,
There was a weak but significant negative correlation between the mean percentage of neutrophils and FEV1 (% predicted) in COPD patients ($p = 0.04$; $r = -0.43$) that was not found in asymptomatic non-smoking PiMZ subjects ($p = 0.55$, $r = 0.05$).

**DISCUSSION**

This study shows for the first time that, in asymptomatic non-smoking PiMZ subjects without airflow obstruction, the number of neutrophils in induced sputum and the level of IL-8 in sputum supernatant are higher than in healthy control subjects and similar to those of patients with COPD. Furthermore, the neutrophil count is related to the levels of cytokines involved in neutrophil chemotaxis. An increased number of neutrophils with a high concentration of cytokines involved in neutrophilic chemotaxis has been previously found in induced sputum of patients with stable COPD and in those with COPD with severe AATD. Moreover, using spontaneous sputum and BAL, an increase in both the airway neutrophil burden and soluble neutrophilic chemoattractants IL-8 and LTB4 was also shown in AATD subjects with airflow obstruction. In this study we found an increased number of neutrophils and a decreased number of macrophages in asymptomatic non-smoking PiMZ subjects without airflow obstruction, similar to that observed in patients with stable COPD. These data suggest that the increased number of neutrophils might have a potential role in the deterioration in lung function, promoting a cascade of inflammatory events in the airways of asymptomatic PiMZ subjects as a result of an impaired equilibrium in the balance between proteases and antiproteases.

The lung neutrophil burden in PiMZ subjects was correlated with high levels of IL-8 in sputum supernatant as previously observed in PiZZ AATD subjects with COPD. A possible explanation for the source of neutrophil infiltration and IL-8 in PiMZ subjects is the presence in the airways of IL-8 in PiMZ subjects without lung impairment may be induced either by increased neutrophilic recruitments or by the secretion from the bronchial epithelium and alveolar macrophages, as a direct effect of uninhibited free elastase. IL-8 may also be produced in airway epithelial cells in order to induce chemotaxis and activation of neutrophils and of eosinophils after exposure with different stimuli such as viral and/or bacterial infections. However, our PiMZ subjects were asymptomatic and we may clinically rule out the potential role of respiratory viral or bacterial infections in influencing our results.

Furthermore, Lomas et al have recently shown the formation of AAT polymers in the lungs of subjects with AATD due to the Z mutation. This polymerisation not only inactivates AAT but also converts the molecule into a powerful proinflammatory chemoattractant agent for human

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic and functional characteristics and AAT blood levels of study subjects</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PiMZ (n = 10)</td>
</tr>
<tr>
<td>M/F</td>
<td>5/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.8 (9.7)</td>
</tr>
<tr>
<td>AAT (mg/dl)</td>
<td>108 (31.7)</td>
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<tr>
<td>FEV1 (% pred)</td>
<td>112.9 (12.2)</td>
</tr>
<tr>
<td>FEV1/FVC (% pred)</td>
<td>88.5 (6.7)</td>
</tr>
<tr>
<td>Tlco (% pred)</td>
<td>103.5 (4.0)</td>
</tr>
<tr>
<td>Smoking history</td>
<td>Smokers 0</td>
</tr>
<tr>
<td></td>
<td>Never smokers 10</td>
</tr>
</tbody>
</table>

AAT, alpha-1-trypsin; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; Tlco, carbon monoxide transfer factor.

Data are expressed as mean (SD).

* $p = 0.05$.

**Table 2 | Sputum induction findings**

<table>
<thead>
<tr>
<th></th>
<th>PiMZ (n = 10)</th>
<th>COPD (n = 10)</th>
<th>Controls (n = 10)</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils (%NSC)</td>
<td>69.3 (18.2)</td>
<td>76.3 (9.9)</td>
<td>45.1 (7.2)*</td>
<td>0.000</td>
</tr>
<tr>
<td>$n \times 10^9/$rel</td>
<td>84.5 (22.2)</td>
<td>126.9 (18.8)</td>
<td>55.0 (8.7)*</td>
<td>0.000</td>
</tr>
<tr>
<td>Macrophages (%NSC)</td>
<td>26.8 (9.4)</td>
<td>18.5 (11.3)</td>
<td>50.5 (5.6)</td>
<td>0.000</td>
</tr>
<tr>
<td>$n \times 10^9/$rel</td>
<td>32.7 (11.5)</td>
<td>30.1 (21.4)</td>
<td>61.6 (9.2)*</td>
<td>0.000</td>
</tr>
<tr>
<td>Eosinophils (%NSC)</td>
<td>0.6 (1.0)</td>
<td>2.4 (2.9)</td>
<td>1.2 (1.3)</td>
<td>0.103</td>
</tr>
<tr>
<td>$n \times 10^9/$rel</td>
<td>0.7 (1.2)</td>
<td>3.8 (5.5)</td>
<td>1.4 (1.5)</td>
<td>0.083</td>
</tr>
<tr>
<td>Lymphocytes (%NSC)</td>
<td>3.1 (1.1)</td>
<td>2.6 (2.2)</td>
<td>2.4 (1.8)</td>
<td>0.971</td>
</tr>
<tr>
<td>$n \times 10^9/$rel</td>
<td>3.7 (1.3)</td>
<td>3.9 (4.1)</td>
<td>2.9 (2.1)</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-8 (ng/ml)</td>
<td>828.5 (490.6)</td>
<td>882.5 (524.3)</td>
<td>3.5 (0.5)*</td>
<td>0.000</td>
</tr>
<tr>
<td>Median</td>
<td>1003.0</td>
<td>934.9</td>
<td>3.5</td>
<td>0.000</td>
</tr>
<tr>
<td>Range</td>
<td>1260–1000</td>
<td>1506–258</td>
<td>4.5–2.5</td>
<td>0.000</td>
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</table>

Data are expressed as mean (SD).

* $p = 0.000$ v PiMZ and COPD (ANOVA with Bonferroni correction).
neutrophils. These polymers can form spontaneously within the lungs of subjects with Z AAT related emphysema (PiZZ), with increased release of LTB4 secreted from alveolar macrophages and high levels of LTB4 and IL-8 in induced sputum compared with subjects with normal AAT levels. It is possible that the chemotactic properties of polymeric AAT may provide a further explanation for the excessive neutrophil burden also found in the lungs of PiMZ subjects before the development of airflow obstruction, and theoretically Z polymer formation might play a role in the pathogenesis of COPD in some MZ heterozygotes. This has recently been proposed as a potential mechanism of airway inflammation in PiMZ subjects, although it has not been proved. Further studies assessing the presence of AAT polymers in the lungs of PiMZ subjects could confirm this hypothesis. Based on these results, we can speculate that the process leading to lung destruction and emphysema in AATD might be related not only to the destruction of connective tissue due to uninhibited free elastase but also to the greater degree of persistent neutrophilic inflammation in the airways and lung parenchyma of MZ heterozygotes.

It is noteworthy that PiMZ subjects were asymptomatic despite the presence of neutrophil infiltration similar to that of COPD patients. This could be explained by the fact that PiMZ subjects were non-smokers and also that were younger than COPD patients. In the patients with COPD there was a significant inverse correlation between the percentage of neutrophils and FEV1 (% predicted). In line with other reports, this finding indicates that the severity of airflow obstruction in COPD seems to be influenced by the amount of chronic neutrophilic recruitment. Conversely, in non-smoking asymptomatic PiMZ subjects, the neutrophil burden was not related to FEV1 (% predicted), suggesting an early and low grade persistent inflammatory process which could accelerate lung destruction in the presence of severe AATD or smoking. Our healthy control subjects were age matched with PiMZ subjects but not with COPD patients. We know that the cellular pattern in sputum is age related and, on this basis, we strongly point out the long term clinical relevance of the early inflammatory status in the airways of PiMZ subjects.

We included COPD patients as a reference group for sputum neutrophilia in order to identify similarity in airway inflammation between PiMZ subjects without airflow obstruction and COPD patients with irreversible airflow limitation. On the other hand, we decided to exclude PiZZ patients with airflow limitation because airway neutrophilic inflammation in these subjects has already been demonstrated both in BAL fluid and in sputum analysis. Although there is a substantial debate within the scientific community as to whether the heterozygous condition of AATD is associated with an increased risk for COPD, we showed that in a group of PiMZ AATD subjects without airflow obstruction a neutrophilic airway inflammation is present. A limitation of this study might be the lack of dosage of free elastase in the airways of PiMZ subjects. We believe, however, that the present data represent a novel and relevant contribution to a better understanding of the biology of the lung of PiMZ subjects. Further large studies are needed to evaluate other important parameters such as elastase to make clear the natural history of PiMZ subjects.

In conclusion, our results indicate that asymptomatic non-smoking PiMZ subjects without airflow obstruction exhibit a significant number of neutrophils in the induced sputum comparable to that observed in COPD patients, reflecting an early and persistent low grade of airway inflammation due to neutrophilic recruitment which is probably sustained by chemotactic factors. Such findings, together with the deficiency of antiprotease activity, might explain the progression towards the obstructive lung disease observed in some PiMZ subjects.

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Conflict of interest: none declared.

REFERENCES
LUNG ALERT

Obstructive sleep apnoea and risk of stroke and death


The effect of obstructive sleep apnoea (OSA) as a risk factor for stroke and death was investigated in 1022 referrals to the Yale Center for Sleep Medicine. 68% (697 subjects) had OSA as defined by an apnoea-hypopnoea index (AHI) of more than five events per hour. The mean AHI in the patients with OSA was 35 while the mean index for the control group was 2.

Data on stroke and death from any cause was obtained for 842 patients (82%). More than half the patients with OSA were being treated with positive airway pressure. In the group with OSA there were 22 strokes and 50 reported deaths (3.48 events per 100 person years), compared with two strokes and 14 deaths in the control group (1.60 events per 100 person years). After adjustment for age, sex, race, smoking status, alcohol consumption, body mass index, diabetes mellitus, hyperlipidaemia, atrial fibrillation and hypertension, OSA remained statistically associated with an increased risk of stroke and death (hazard ratio 1.97; 95% CI 1.12 to 3.48, \( p = 0.01 \)). Increased severity of OSA was associated with an increased risk of developing this composite end point (\( p = 0.005 \)).

This study shows that OSA is significantly associated with the risk of stroke and death, independently of other risk factors.

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