

ALPHA-1-ANTITRYPSIN DEFICIENCY

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

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Thorax 2006;61:129-133. doi: 10.1136/thx.2005.043471

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Received 7 March 2005
Accepted 30 October 2005
Published Online First
24 November 2005

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 ($84.5 (22.2) \times 10^4/\text{ml}$) and patients with COPD ($126.9 (18.8) \times 10^4/\text{ml}$) than in matched normal subjects ($55.0 (8.7) \times 10^4/\text{ml}$). IL-8 levels were increased in PiMZ subjects ($828.5 (490.6) \text{ ng/ml}$; median 1003.0 ng/ml ; range $1260\text{--}100 \text{ ng/ml}$) and in COPD patients ($882.5 (524.3) \text{ ng/ml}$; median 934.9 ng/ml ; range $1506\text{--}258 \text{ ng/ml}$) compared with normal subjects ($3.5 (0.5) \text{ ng/ml}$; median 3.5 ng/ml ; range $4.5\text{--}2.5 \text{ ng/ml}$). 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.

Alpha-1-antitrypsin (AAT) is a serine protease inhibitor that protects the lung from the proteolytic action of proteases such as neutrophil elastase. Severe alpha-1-antitrypsin deficiency (AATD), a genetic disorder due to homozygosity for the protease inhibitor (Pi) Z allele (PiZZ) and characterised by low AAT serum concentrations, is recognised as a risk factor for chronic obstructive pulmonary disease (COPD) with the typical pathological findings of premature panacinar emphysema dependent on the protease-antiprotease imbalance.¹ Heterozygotes for the Z allele (most commonly PiMZ) have lower serum levels of AAT than normal individuals (PiMM),² but the role of intermediate deficiency PiMZ of developing COPD remains uncertain.³ Sputum induction is a direct, non-invasive, and reproducible method for assessing airway inflammation.^{4,5} Previous studies have demonstrated a predominance of neutrophils and related chemokines such as interleukin-8 (IL-8) and leukotriene B4 (LTB4) in the induced sputum of patients with COPD.^{6,7} Airway neutrophilic inflammation plays a fundamental role in the pathogenesis and progression of COPD. In the sputum of subjects with severe AATD with airflow obstruction Stockley *et al*⁸ have shown a pattern of airway inflammation similar to that of COPD patients. In addition, Rouhani *et al* observed an increased lung neutrophil burden related to pro-inflammatory cytokines such as IL-8 and IL-6 in bronchoalveolar lavage (BAL) fluid of AATD subjects with mild functional lung impairment.⁹ The increased neutrophil recruitment is thought to contribute further to the development of the rapidly progressive lung deterioration observed in subjects with AATD. The aim of this

study was to assess by sputum induction the cellular pattern and IL-8 levels in the airways of non-smoking asymptomatic PiMZ subjects with normal lung function in comparison with COPD patients and healthy control subjects.

METHODS

Study population

AAT deficiency

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 $108 (31.7) \text{ mg/dl}$) 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-tryptsin; AATD, alpha-1-tryptsin 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 criteria¹⁰: 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).¹¹ AAT phenotyping was performed by isoelectric focusing.¹²

Pulmonary function tests

Lung function was measured in accordance with the American Thoracic Society (ATS) standard procedure.¹³ The patients underwent spirometry to measure FEV₁, FVC, and the FEV₁/FVC ratio. The lung carbon monoxide transfer factor (TLco) was assessed by the single breath method (PF/DX system MCG, MN, USA) with patients in the sitting position.

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 Ultraneb 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 Burkert'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 $\times 10^4$ /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 reported 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 ($p = 0.002$; ν COPD $p = 0.046$; ν 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 TLco values between the groups ($p = 0.09$; COPD ν PiMZ $p = 0.26$; COPD ν 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) $\times 10^4$ /ml; 69.3 (18.2) %NSC) and in COPD patients (126.9 (18.8) $\times 10^4$ /ml; 76.3 (9.9) %NSC) than in healthy control subjects (55.0 (8.7) $\times 10^4$ /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) $\times 10^4$ /ml; 26.8 (9.4) %NSC) and in patients with COPD (30.1 (21.4) $\times 10^4$ /ml; 18.5 (11.3) %NSC) than in healthy control subjects (61.6 (9.2) $\times 10^4$ /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.¹⁴ 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 ($p = 0.036$; $r = 0.66$,

Table 1 Demographic and functional characteristics and AAT blood levels of study subjects

	PiMZ (n = 10)	COPD (n = 10)	Controls (n = 10)	p value
M/F	5/5	6/4	4/6	0.67*
Age (years)	47.8 (9.7)	65.7 (13.3)†	46.1 (21.7)	0.019
AAT (mg/dl)	108 (31.7)‡	140.3 (10.2)	162.5 (18.5)	0.002
FEV ₁ (% pred)	112.9 (12.2)	60.8 (23.2)§	107.6 (15.1)	0.000
FEV ₁ /FVC (% pred)	88.5 (6.7)	58.3 (13.6)§	87.7 (8.2)	0.000
TlCO (% pred)	103.5 (4.0)	96.9 (12.6)	104.9 (5.6)	0.09
Smoking history				0.000*
Smokers	0	5	0	
Ex-smokers	0	5	0	
Never smokers	10	0	10	

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).

* χ^2 test.

†Compared with PiMZ subjects (p=0.38); compared with controls (p=0.047) (ANOVA with Bonferroni correction).

‡Compared with COPD patients (p=0.046); compared with controls (p=0.002) (ANOVA with Bonferroni correction).

§Compared with PiMZ subjects and controls (p=0.000) (ANOVA with Bonferroni correction).

fig 1). 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 IL-8 in the sputum of PiMZ subjects, suggesting a role for IL-8 in the recruitment of neutrophils or IL-8 release by activated neutrophils in the airways of PiMZ asymptomatic subjects.

An increased number of neutrophils with a high concentration of cytokines involved in neutrophilic chemotaxis has previously been found in induced sputum of patients with stable COPD^{15–18} and in those with COPD with severe AATD.¹⁹ 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.^{9, 20, 21} 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.^{9, 21} A possible explanation for the source of neutrophil infiltration and IL-8 in PiMZ subjects is the presence in the airways of free elastase. High concentrations of IL-8 in the sputum supernatant observed in PiMZ subjects without lung impairment may be induced either by increased neutrophilic recruitment itself 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^{22, 23} after exposure with different stimuli such as viral and/or bacterial infection.²⁴ 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.²⁵ This polymerisation not only inactivates AAT but also converts the molecule into a powerful proinflammatory chemoattractant agent for human

Table 2 Sputum induction findings

	PiMZ (n = 10)	COPD (n = 10)	Controls (n = 10)	p value
Neutrophils (%NSC)	69.3 (18.2)	76.3 (9.9)	45.1 (7.2)*	0.000
n × 10 ⁴ /ml	84.5 (22.2)	126.9 (18.8)	55.0 (8.7)*	0.000
Macrophages (%NSC)	26.8 (9.4)	18.5 (11.3)	50.5 (7.6)*	0.000
n × 10 ⁴ /ml	32.7 (11.5)	30.1 (21.4)	61.6 (9.2)*	0.000
Eosinophils (%NSC)	0.6 (1.0)	2.4 (2.9)	1.2 (1.3)	0.103
n × 10 ⁴ /ml	0.7 (1.2)	3.8 (5.5)	1.4 (1.5)	0.083
Lymphocytes (%NSC)	3.1 (1.1)	2.6 (2.2)	2.4 (1.8)	0.971
n × 10 ⁴ /ml	3.7 (1.3)	3.9 (4.1)	2.9 (2.1)	0.95
IL-8 (ng/ml)	828.5 (490.6)	882.5 (524.3)	3.5 (0.5)*	0.000
Median	1003.0	934.9	3.5	
Range	1260–100	1506–258	4.5–2.5	

Data are expressed as mean (SD).

*p=0.000 v PiMZ and COPD (ANOVA with Bonferroni correction).

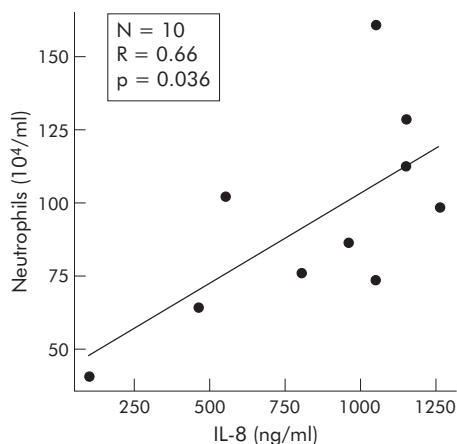


Figure 1 Correlation between supernatant IL-8 concentration and number of neutrophils in the induced sputum of PiMZ subjects.

neutrophils.²⁶ These polymers can form spontaneously within the lungs of subjects with Z AATD 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.²¹ 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,²⁷ 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 they 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.²⁸ 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 related²⁹ 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 fluid⁹ and in sputum analysis.²¹ 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.

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LUNG ALERT

Obstructive sleep apnoea and risk of stroke and death

▲ Yaggi HK, Concato J, Kernan WN, *et al*. Obstructive sleep apnea as a risk factor for stroke and death. *N Engl J Med* 2005;**353**:2034–41

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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