

# Tobacco smoking associated with the increases of the bronchoalveolar levels of interleukin-5 and interleukin-1 receptor antagonist in acute eosinophilic pneumonia

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**Abstract. – BACKGROUND:** There is a strong association between tobacco smoking and acute eosinophilic pneumonia (AEP) and tobacco smoking has been related to onset of AEP, but the mechanisms for causing AEP are largely unknown. AEP is characterized by locally high levels of interleukin-5 (IL-5) and interleukin-1 receptor antagonist (IL-1RA). Thus, tobacco smoking may relate to the high levels of IL-5 and IL-1RA in AEP.

**AIM:** We aimed to determine whether the rate of tobacco smoking positively related to the high levels of IL-5 and IL-1RA.

**PATIENTS AND METHODS:** In the study, 155 AEP patients and 135 control healthy subjects were recruited by the Clinical Research Center at General Hospital of Shenyang Military Area Command (Shenyang, China). All the subjects were interviewed regarding for the tobacco smoking rate. The number of eosinophils was counted in all subjects. The relative expressive levels of IL-5 and IL-1RA were measured with ELISA in Bronchoalveolar lavage (BAL) and serum.

**RESULTS:** The BAL levels of IL-5, IL-1ra, eosinophils and lymphocytes positively correlates with the rate of tobacco smoking in AEP patients ( $p < 0.005$ ) comparing with those in healthy control groups. No significant association was observed between the serum levels IL-5 and IL-1ra and the rate of tobacco smoking in AEP patients ( $p > 0.05$ ) comparing with those in healthy control groups.

**CONCLUSIONS:** Smoking increases the BAL levels of IL-5 and IL-1RA, which is associated with the onset of AEP.

## Key Words:

Bronchoalveolar lavage (BAL), Interleukin-5 (IL-5), Interleukin-1 receptor antagonist (IL-1RA), Acute eosinophilic pneumonia (AEP), Tobacco smoking.

## Introduction

Acute eosinophilic pneumonia (AEP) is a sudden and severe febrile illness that can result in life-

threatening respiratory failure<sup>1</sup>. This disease can mimic other diseases causing acute respiratory failure. The characteristics of AEP are diffuse bilateral radiographic infiltrates, pulmonary eosinophilia, severe hypoxemia and fever. The disease is an infrequent disease and seldom published. Thus, the diagnosis may be missed or delayed. It is clinically distinguishable from other pulmonary eosinophilic diseases and the examination of bronchoalveolar lavage (BAL) can be an assistant method in diagnosis in most cases<sup>1</sup>. Treatment with corticosteroids results in rapid reversal of respiratory failure and complete recovery, generally without relapse. In some patients, most researches support the idea that AEP may be caused by tobacco smoking<sup>2-5</sup>; Tobacco smoking has been associated with impaired pulmonary functions and increased appearance of infections<sup>6,7</sup>. There is the association between eosinophilic inflammation and AEP<sup>8</sup>, but the mechanism behind the phenomenon is still mostly unclear.

Interleukin-5 (IL-5) and Interleukin-1 receptor antagonist (IL-1RA) are proinflammatory cytokines that produced from lymphocytes<sup>9,10</sup>. The patients with AEP had high BAL levels of IL-5, IL-1RA. In the serum of patients with AEP, IL-5 was not detected, and IL-1RA was initially high but fell after corticosteroid treatment<sup>11</sup>. IL-5 is required for activation and recruitment of eosinophils to the lung and it is eosinophils that are thought to account for most lung disease induced by IL-5<sup>12</sup>. IL-1 is a cytokine involved in the initiation and amplification of the defense response in infectious and inflammatory diseases. IL-1RA is an inactive member of the IL-1 family and represents one of the most potent mechanisms for controlling IL-1-dependent inflammation. IL-1RA is effective in the therapy of acute and chronic inflammatory diseases in experimental animal models and also in preliminary clinical trials<sup>13</sup>. We conjecture that tobacco smoke causing

AEP may relate to the increased level of IL-5 and IL-1RA in BAL. The aim of the study was to determine levels of inflammatory markers in AEP patients and healthy subjects; and to analyze possible relations with tobacco smoking rate.

## Patients and Methods

### Participants

From March 7<sup>th</sup>, 2009 to March 6<sup>th</sup>, 2012, a total of 155 patients were recruited by the Clinical Research Center at General Hospital of Shenyang Military Area Command (Shenyang, China). Among them, 99 were recruited retrospectively while 56 were recruited prospectively. At the same period, 135 healthy subjects were recruited.

The diagnostic requirements were included in the AEP group. The causes of pulmonary eosinophilia by drugs, parasites or toxins were excluded from the group. The diagnostic criteria for AEP include recent onset (less than 1 month) of pulmonary symptoms, respiratory failure ( $\text{PaO}_2 < 60$  while breathing room air), diffuse pulmonary infiltrates on chest x-ray film, a high percentage of BAL eosinophil (generally greater than 25%). Peripheral blood was tested and routine coproparasitological study for lung parasites was performed, showing that the absence of any identifiable infectious cause of the symptoms, and a prompt, complete response to corticosteroids. For all healthy subjects, they have normal chest radiographic findings and are free of the pulmonary symptoms as above mentioned. Additionally, clinical characteristics of AEP patients and healthy subjects were compared in the study: (1) Mean and standard deviation of: age,  $\text{PaO}_2/\text{FiO}_2$  ratio and serum and BAL eosinophilia, lymphocytes, IL-5 and IL-1RA levels for AEP and healthy patients, with their respective *p* value from Student's *t*-test. (2) The number of patients that need mechanical ventilation, and the percentage of patients presenting with the most frequent symptoms (cough, dyspnea, fever, etc) for AEP patients. (3) The association between

higher levels of Interleukins in BAL and hospitalization rates was assessed. (4) Lung infiltration score in lung CT was compared between AEP patients and healthy control group.

The patients with AEP and healthy subjects were stratified in four groups according to the tobacco smoking rate as Table I showed. Smoking used to be considered more of a male pursuit with much higher numbers of men smoking than women. In China, for example, 61% of men are reported to be current smokers, compared with only 4.2% of women<sup>14</sup>, so only males were considered here. We present the case of from 50-year-old to 64-year-old males with personal histories of tobacco habit of from 0 to 15 cigarettes/day (the same brand for the cigarettes) for the last 10 years. Analytical studies showed normal arterial blood gases, immunoglobulins and alpha-1-antitrypsin. Ethics approval was obtained from the General Hospital of Shenyang Military Area Command. All the patients were checked for eosinophil, lymphocytes, IL-5 and IL-1RA levels before corticosteroids treatment.

### Blood Sample Collection and BAL Procedure

Both serum samples were obtained from AEP patients and healthy volunteers. Peripheral venous blood samples were taken and stored at  $-80^\circ\text{C}$  immediately. BAL was obtained using a flexible fiberoptic bronchoscope (Olympus 1T-200; Olympus, Tokyo, Japan) after local anesthesia of the upper airways with 4% lidocaine. The bronchoscope was wedged into one of the segmental bronchi of the right middle lobe and 50 ml sterilized saline was then instilled through the bronchoscope. The fluid was immediately retrieved by gentle suction using a sterile syringe and the procedure was repeated three times. BAL was passed through two sheets of gauze and then centrifuged at 500 g for 10 min at  $4^\circ\text{C}$ . After washing twice with PBS, the remaining cells were suspended in PBS

**Table I.** Number of subjects per stratified group according to smoking rate.

| Healthy subjects and AEP patients | Cigarettes/day | The number of healthy subjects | The number of acute eosinophilic pneumonia |
|-----------------------------------|----------------|--------------------------------|--------------------------------------------|
| Non-smoker                        | 0              | 30                             | 30                                         |
| Light-smoker                      | 0.1-5.0        | 30                             | 40                                         |
| Middle-smoker                     | 5.1-10.0       | 45                             | 40                                         |
| Heavy-smoker                      | 10.1-15.0      | 30                             | 45                                         |

(phosphate-buffered saline) supplemented with 10% heat inactivated fetal calf serum and counted using a haemocytometer. An aliquot was then diluted to a concentration of  $2 \times 10^5$  cells/ml and a 0.2 ml cell suspension was spun down onto a glass slide at 1100 rpm for 2 min. The remaining fluid was centrifuged at 500 g for 5 min and the supernatant was stored at  $-80^\circ\text{C}$  until examined. The slides were dried, fixed, and then stained by the May-Giemsa method.

#### **Measurement of the Expression Levels of IL-5, IL-1RA and IFN- $\beta$**

The levels of IL-5, IL-1RA and IFN- $\beta$  were measured by commercial kits according to the protocols provided by the manufacturer. IL-5 and IL-1RA concentrations in the fivefold-diluted serum and the BAL were measured with an ELISA (Human IL-5 and IL-1RA ELISA Kit, Sino Biological Inc, Beijing, China). IFN- $\beta$  concentrations in the serum and the BAL were also measured with an ELISA (IFN- $\beta$  EASIA; BioSource Europe, Nivelles, Belgium).

#### **Statistical Analysis**

Differences between two groups were compared by one-way analysis of variance (ANOVA). We used Spearman's rank correlation coefficient to identify the association among IL-5 and IL-1RA levels in serum and BAL samples and the tobacco smoking rate for AEP and healthy subjects. Spearman's rank correlation was also

used for eosinophils and lymphocytes counts. Data were analyzed using Statview 5.0 software (Abacus Systems, Berkeley CA, USA) with a  $p$  value of  $< 0.05$  accepted as significant.

## **Results**

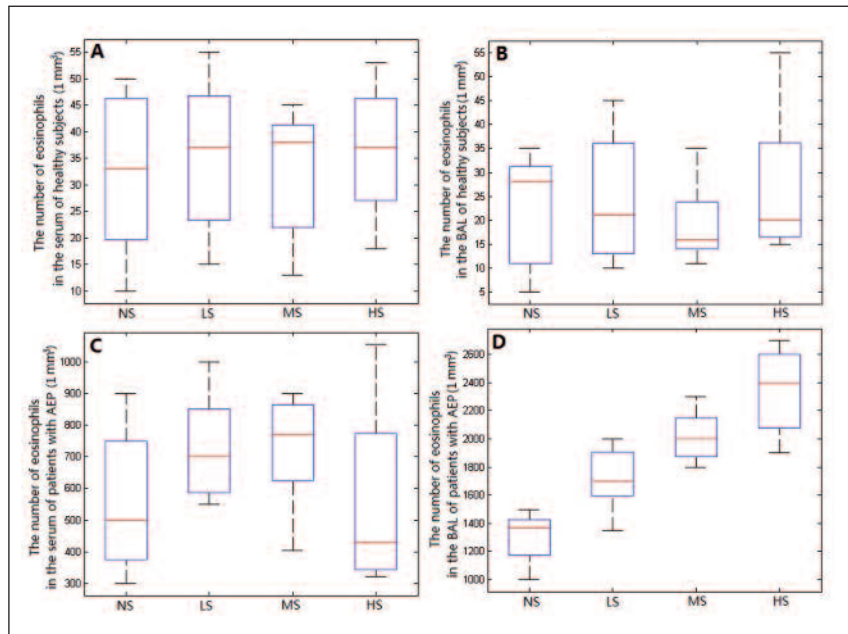
### ***The Clinical Characteristics of AEP Patients and Healthy Subjects***

To reduce the other factors interference, the similar ages between AEP patients and healthy subjects were chosen as Table II showed. The median and data range of  $\text{PaO}_2/\text{FiO}_2$  ratio were significantly lower than those in healthy subjects ( $p < 0.001$ ). The data of BAL eosinophilia, lymphocytes, IL-5 and IL-1RA levels were significantly higher than those in healthy control groups ( $p < 0.001$  for the former three parameters and  $p < 0.01$  for the last one parameter). The data of serum eosinophilia and IL-5 levels were significantly higher than those in healthy control groups ( $p < 0.001$  for the former parameter and  $p < 0.05$  for the latter parameter) (Table II). More than 70% (110 cases in 155 patients) of AEP patients need mechanical ventilation, and the percentage of patients presenting with the most frequent symptoms (cough, dyspnea, fever, etc) for AEP patients was 92%. The levels of Interleukins in BAL and hospitalization rates were positively related. Higher lung infiltration score in lung CT was observed in AEP patients comparing with healthy control group (Table II).

**Table II.** Clinical characteristics of AEP patients and healthy subjects.

|                              |                                        | AEP                  | Healthy subjects |
|------------------------------|----------------------------------------|----------------------|------------------|
| Serum level                  | Age                                    | 55 (50-61)           | 57 (53-64)       |
|                              | $\text{PaO}_2/\text{FiO}_2$ ratio      | 145 (120-190)***     | 379 (320-470)    |
|                              | Eosinophilia (number/mm <sup>3</sup> ) | 620 (300-1060)***    | 19 (5-55)        |
|                              | Lymphocytes (number/mm <sup>3</sup> )  | 950 (550-1390)       | 920 (570-1350)   |
|                              | IL-5 (pg/ml)                           | 35 (8-85)*           | 43 (18-105)      |
| BAL level                    | IL-1ra (pg/ml)                         | 540 (200-1500)       | 580 (90-1160)    |
|                              | Eosinophilia (number/mm <sup>3</sup> ) | 1860 (1000-2700)***  | 20 (5-50)        |
|                              | Lymphocytes (number/mm <sup>3</sup> )  | 1240 (890-1680)**    | 450 (198-695)    |
|                              | IL-5 (pg/ml)                           | 410 (88-1100)**      | 205 (35-470)     |
|                              | IL-1ra (pg/ml)                         | 14000 (3500-26500)** | 680 (220-1280)   |
| NMV (number in AEP patients) |                                        | 110                  | 0                |
| PMFS (%)                     |                                        | 92                   | 0                |
| Lung infiltration score      |                                        | 5.5 (1-11)           | 0                |

For definition of abbreviations: NMV, the number of patients that need mechanical ventilation for AEP patients; PMFS, the percentage of patients presenting with the most frequent symptoms (cough, dyspnea, fever, etc) for AEP patients. Data are presented as median (ranges). \* $p < 0.05$ , compared with healthy volunteers. \*\* $p < 0.01$ , compared with healthy volunteers. \*\*\* $p < 0.001$ , compared with healthy volunteers.



**Figure 1.** Box-and-whisker plots of eosinophils counts in healthy subjects and AEP patients. The red bars in the boxes were average numbers and the boxes represented 95% of the samples. The error bars were above or below the boxes.

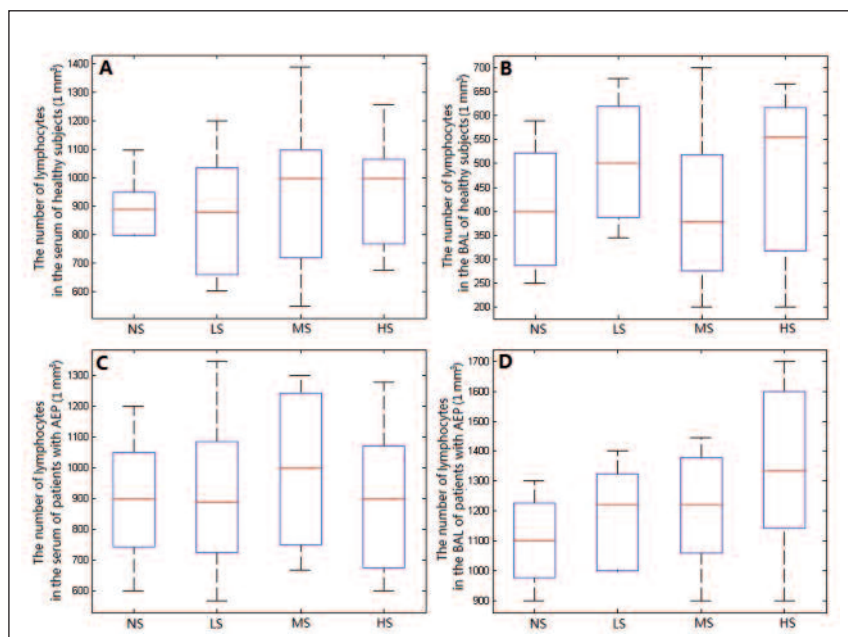
### ***The Number of Eosinophil and Lymphocytes in Serum and BAL***

In healthy subjects, there were no significant statistical difference for the average number of eosinophils in serum and BAL (Figure 1A and 1B). For the patients with AEP, the total cell counts and the percentages of eosinophils were significantly higher than those in healthy subjects in both serum and BAL ( $p < 0.01$ ) (Figure 1). For lymphocytes, the number was similar in the

serum between healthy subjects and AEP patients (Figure 2A and 2C).

### ***IL-5 and IL-1RA Levels in Serum and BAL***

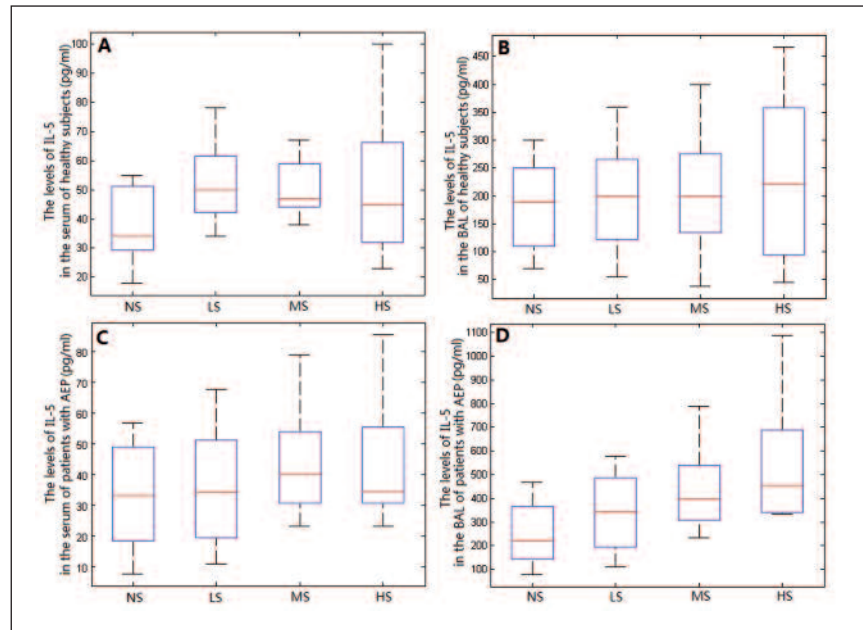
Cytokines are produced in the lung by local resident cells such as alveolar macrophages, lung epithelial cells, and fibroblasts or by cells such as eosinophil, lymphocytes and platelets as a response to local or systemic injury. BAL and serum levels of the studied cytokines on admis-



**Figure 2.** Box-and-whisker plots of lymphocytes counts in healthy subjects and AEP patients. The red bars in the boxes were average numbers and the boxes represented 95% of the samples. The error bars were above or below the boxes.



**Figure 3.** Box-and-whisker plots of the relative expression levels of IL-5 in the serum and BAL from healthy subjects and AEP patients. The red bars in the boxes were average expression levels and the boxes represented 95% of the samples. The error bars were above or below the boxes.



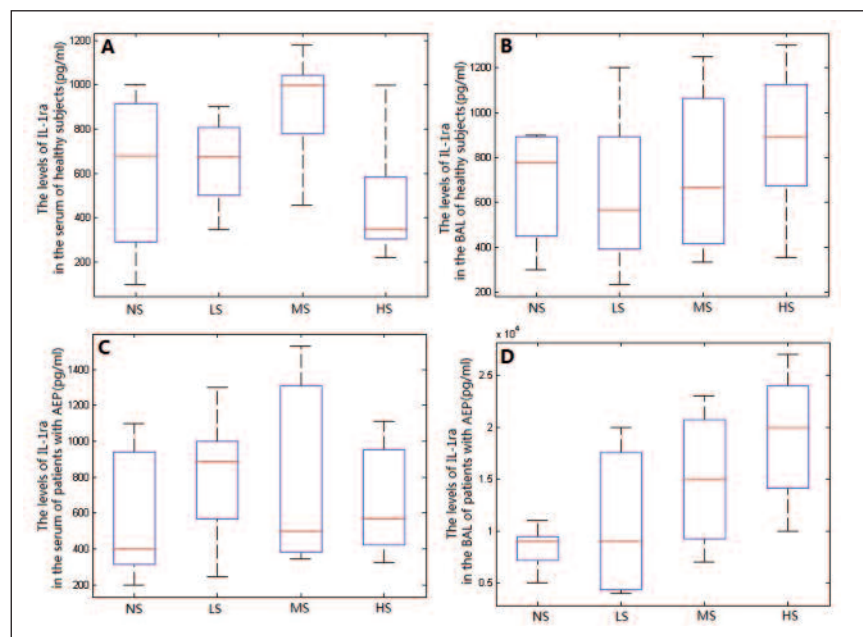
sion may provide valuable information for lung disease development in the patients at risk<sup>15</sup>. Thus, the work was performed here.

The median BAL levels of IL-5 and IL-1RA in were correlated with the percentage of eosinophils and lymphocytes (Figure 1D, 2D, 3D and 4D) in the BAL of the patients with AEP. The BAL level of IFN- $\beta$  did not differ (all  $p > 0.05$ ) among the all groups (data were not shown).

#### ***The Association Between the Degrees of Tobacco Smoking and the Levels of IL-5 and IL-1RA***

We guessed that AEP may be caused by the elevated level of IL-5 and IL-1RA according to previous report<sup>11</sup>, so we investigated whether smoking might affect the expression levels of IL-5 and IL-1RA in AEP patients. In the AEP patients, our observations did not reveal any influence of smoking rate on the levels of IL-5 and IL-1RA in the

**Figure 4.** Box-and-whisker plots of the relative expression levels of IL-1RA in the serum and BAL from healthy subjects and AEP patients. The red bars in the boxes were average expression levels and the boxes represented 95% of the samples. The error bars were above or below the boxes.



serum samples (all  $p > 0.05$ ) (Figure 3C and 4C) comparing with the healthy control groups. The Spearman's rank correlation coefficient for the association between the smoking rate and the levels of IL-5 and IL-1RA in the serum was 0.03 and 0.08 respectively. Thus, to find the function of smoking rate for the onset of AEP, we further explore the effects of tobacco smoking on the BAL levels of interleukins. With change of the tobacco smoking rate from non-smoker to heavy-smoker, the median BAL levels of IL-5 and IL-1RA were increasing ( $p < 0.005$ ) (Figure 3D and 4D) when comparing with healthy subjects. The Spearman correlation for the association between the tobacco smoking rate and median BAL levels of IL-5 or IL-1RA was 0.80 and 0.92 respectively. Therefore, the concentration of IL-5 and IL-1RA in BAL were positively associated with the tobacco smoking rate. Meanwhile, tobacco smoking could also cause the increase of eosinophils and lymphocytes in the BAL of the patients with AEP (Figure 1D and 2D). Taken together, there were significant correlations between the levels of IL-5 and IL-1RA in BAL and tobacco smoking rate (Figure 3D and 4D). The rho value between the levels of IL-5 and IL-1RA in BAL and the degrees of tobacco smoking was 0.80 and 0.92, which were statistically strong associated with AEP.

## Discussion

AEP is a sudden and severe febrile illness that can result in life-threatening respiratory failure<sup>1</sup>. Clinical diagnosis is important for correct treatment. Acute eosinophilic pneumonia is essentially diagnosed by increased number of eosinophils in the BAL<sup>16</sup> and the presence or absence of peripheral-blood eosinophilia is probably not helpful in recognizing AEP<sup>17</sup>. Right diagnosis is crucial but can still be a "major problem" confounded by sampling problems at the time of biopsy<sup>18</sup>. In all the persons with AEP, the median BAL levels of IL-5 and IL-1RA were significantly higher than those in the serum samples ( $p < 0.001$ ) (Figure 3C, 3D, 4C and 4D). However, the levels of some cytokines could not be detected in serum sometimes, which might be affected by the human clinical and metabolic condition. From our results, IL-5 and IL-1RA can be combined adjuvant biomarkers for AEP diagnosis. IL-5 and IL-1RA can be target molecules in AEP, which was consistent with previous reports<sup>11</sup>. The positive values to IL-5 and IL-1RA immunological activity can be an ancillary diagno-

sis of AEP. Furthermore, in the BAL, the number of the lymphocytes of the patients with AEP was significantly higher than those in healthy subjects (Figure 2B and 2D). Thus, AEP can be characterized by the high levels of predominant inflammatory cells eosinophil and lymphocytes in BAL.

Cigarette smoking and exposure to environmental tobacco smoke increase the risk of certain infections<sup>19-22</sup>. Smokers account for approximately half of healthy adult patients with invasive pneumococcal disease<sup>23,24</sup>. We conjectured that smoking is associated with the high expression levels of IL-5 and IL-1RA since the expression levels of IL-5 and IL-1RA increase in the BAL of AEP patients. The high levels of interleukins may also be the result of an indirect mechanism trigger by tobacco and/or other factors. Serum concentration of IL-5 could not be detected in some times. Serum concentrations of IL-1RA were not correlated with smoking status, which was also consistent with previous report<sup>25</sup>. However, we found the change for the levels of IL-5 and IL-1RA could be detected in BAL since BAL proved to be a useful method to permit sampling of cells from alveolar areas. The levels of IL-5 and IL-1RA in BAL were significantly affected by the tobacco smoking rate, suggesting smoking increased the risk of hospitalization for AEP by increasing the BAL levels of IL-5 and IL-1RA. The association between the degrees of tobacco smoking and the levels of IL-5 and IL-1RA in BAL provided indirect support for a smoking pathogenesis hypothesis in AEP patients by increasing IL-5 and IL-1RA levels in BAL instead of serum samples.

The clinical characteristics and association of AEP to smoking habits has been already reported. The majority of the patients altered their smoking habits within a median (interquartile range) of 17 (13-26) days prior to development of AEP<sup>26</sup>. The strong association between smoking and AEP onset, particularly recent change in smoking habits (reported in 91% of patients) should be addressed here. A hypothesis of interleukins involve in the genesis of AEP could be made, but there is not clear knowledge of the physiopathology of this disease up to date.

## Conclusions

Here, we investigated 155 AEP patients and 135 healthy subjects for studying the effects of smoking rate on the onset of AEP. Our data, tobacco smoking rate positively relating to the higher BAL levels of IL-5 and IL-1RA, which are strong-

ly associated with the risk of AEP. Thus, IL-5 and IL-1RA can be novel targets for curing AEP. Certainly, this study is lacking of follow up of patients to determine if higher interleukin or eosinophils levels correlate to AEP severity or outcome. Therefore, further work need to be done to address such problems in future.

### Conflict of interest

The Authors declare that they have no conflict of interests.

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