

## Nasal Eosinophils Display the Best Correlation with Symptoms, Pulmonary Function and Inflammation in Allergic Rhinitis

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### Key Words

Allergic rhinitis · Correlation · Methacholine · Nasal eosinophils · Spirometry · Symptoms

### Abstract

**Background:** The pathogenesis of allergic rhinitis and its link with asthma are well known. Nevertheless, a complete cross-sectional evaluation of the usually available clinical, functional and immunological parameters has never been made. We assessed nasal symptoms and flow, cytology, cytokines, pulmonary function and methacholine positivity in a large number of patients with pure pollinosis. **Methods:** Young men presenting at a military hospital for routine follow-up were recruited for the study. They had to suffer from rhinitis alone (without asthma) for at least 2 years and had to have a positive skin prick test to pollens only. During the pollen season, they underwent symptom evaluation, measurement of nasal flow, nasal scraping and lavage (cell count and assay for IL-4, IL-5, IL-8 and IFN $\gamma$ ), pulmonary function tests and methacholine challenge. **Results:** Fifty subjects ( $23.7 \pm 4.9$  years old) were enrolled. All patients had high clinical scores ( $9.5 \pm 1.6$ ) and inflammatory cells (eosinophils:  $10.5 \pm 4$  and neutrophils  $21.3 \pm 6$ ) and low

nasal flow ( $482 \pm 111$  ml/s). We found that the number of eosinophils in nasal scrapings highly correlated with all the above-mentioned parameters, including nasal flow, cytokines and spirometric values. A significant positive correlation was found between all inflammatory cells and all cytokines. IL-8, IL-4 and neutrophils displayed only a partial correlation with pulmonary parameters (FEV<sub>1</sub>, FVC and FEF<sub>25–75%</sub>), at variance with IL-5 and eosinophils. Methacholine test positivity significantly correlated with the number of eosinophils in the nasal smear. **Conclusion:** Eosinophils in the nasal smear display the best correlation with all the clinical and immunological parameters in allergic rhinitis and also correlate well with methacholine response.

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### Introduction

Allergic rhinitis (AR) is probably the most common immunological disease. Based on large epidemiological studies, its prevalence is estimated to be around 10–20% [1, 2], and it is constantly increasing during the last decades [3]. Although AR is not a life-threatening condition, its socio-economic effects are substantial, when direct

and indirect costs are taken into account [4]. Moreover, AR has often been associated with asthma and represents a risk factor for the development of asthma itself [5].

The interaction between allergens and IgE-mast cells plays a triggering role, but the immediate phase is followed by a delayed-type inflammatory phase and, if the allergenic stimulus persists, a chronic and sustained inflammation ensues [6]. Allergen exposure activates mast cells resulting in the release of mediators and cytokines capable of inducing inflammatory cell recruitment (including eosinophils, neutrophils, and Th2 cells) and their activation at the target organ level [7]. The immunopathologic features of AR have been related to the effects of Th2-derived cytokines. Increased levels of Th2-derived cytokines, including IL-3, IL-4, IL-5, IL-13, and GM-CSF, have been extensively described in AR [8]. From a clinical viewpoint, the histamine-mediated reaction is clinically expressed by sneezing, itching, rhinorrhea and congestion, whereas nasal obstruction is predominantly sustained by inflammation.

Another important aspect of AR is its strict association with asthma, since the upper and the lower respiratory tract are a single morphofunctional unit [9, 10]. Indeed, experimental data obtained *in vivo* have demonstrated that the bronchial allergic response is the same in patients with rhinitis or asthma [11]. Moreover, recent experiments have consistently shown that a bidirectional link operates between upper and lower respiratory airways [12, 13]. Inflammation also accounts, to a certain extent, for non-specific bronchial hyperresponsiveness (BHR) [14].

From a clinical and investigational point of view, it is difficult to quantify the link between asthma and rhinitis, since there is so far no comprehensive parameter evaluating nose and bronchi at the same time. Symptom score and cell count, for instance, are easily assessed in nasal smear, but they provide only little information about the ongoing local inflammatory processes. On the other hand, the amount of inflammatory cytokines in nasal fluid only describes some aspects of the inflammatory process. Concerning the lower airways, an objective estimation of the inflammatory processes would require the use of invasive methods (i.e. fiberoptic bronchoscopy and bronchial biopsy). Nevertheless, BHR, a distinctive feature of asthma, is easy to measure in real life and provides valuable information on the reactivity of the bronchial wall, whereas FEF<sub>25-75%</sub> reflects the involvement of small airways [15].

Pollen-induced rhinitis represents a reliable model to investigate the relationship among clinical parameters,

local inflammation and involvement of the lower airways, since the exposure to the allergen is confined to a short period of time. In order to better clarify these relationships, we assessed different subjective and objective parameters in a large and homogeneous cohort of patients with pure pollinosis at the same time.

## Patients and Methods

### Patients

Adult male conscripts, referred for routine fitness visits to the Navy Military Hospital of La Spezia, were selected based on the presence of pure seasonal rhinitis. Mandatory inclusion criteria were: symptoms of rhinitis (sneezing, itching, rhinorrhea and congestion) with seasonal onset in the preceding 2 years, and a positive skin prick test to one or more of the following: grasses, *Parietaria*, birch, hazel or olive. During the pollen season (April to June), when subjects had symptoms (scored by an ordinal scale), nasal scraping for cell counts, nasal lavage for cytokine measurements, rhinomanometry, pulmonary function tests and bronchial methacholine challenge tests were carried out.

### Diagnosis

The presence of asthma, clinically defined as recurrent attacks of wheezing, chest tightness, breath shortness, and cough (especially during the night or in the early morning) was also investigated. A positive history of asthma, according to GINA [16], was an exclusion criterion. Patients with skin test positivity to dust mites, moulds, cat and dog dander and current or previous smokers were also excluded. Skin tests were applied on the anterior surface of the forearm using 1-mm prick lancets (Stallergenes, Milan, Italy). The skin reaction was recorded after 15 min by evaluating the response in comparison with the wheal of the positive (histamine HCl 0.1%) and the negative (saline) control. A wheal diameter equal or greater than 3 mm was considered positive [17]. Patients meeting the inclusion criteria were invited to a subsequent visit during the pollen season for further assessments.

### Symptom Score

During the clinical visit, performed during the pollen season (May to June) and out of the pollen season, patients were required to score each of their symptoms in the previous 24 h (obstruction, itching, sneezing or rhinorrhea). The severity score was: 0 = absent, 1 = mild (symptom was present but not annoying or troublesome), 2 = moderate (symptom was frequently troublesome but did not interfere with either normal daily activity or sleep) and 3 = severe (symptom was sufficiently troublesome to have interfered with normal daily activity or sleep). A nasal smear and a nasal lavage were performed on that occasion. All patients were required to withdraw antihistamines, nasal steroids or decongestants at least 10 days before the scheduled visit.

### Nasal Cells and Cytokines

For cell counts, nasal specimens were obtained from one nostril by scraping the head of the inferior turbinate with a cotton swab [18]. Briefly, after scraping, the cotton tip was immersed in a 10-ml tube containing phosphate-buffered saline; the fluid recovered was

centrifuged at 220 g per min for 10 min, and the pellet was resuspended in PBS (2 ml). Cell suspensions were filtered to reduce the quantity of mucus, and cytospin slides were prepared. Samples were stained with Diff Quik stain and analyzed using an optic microscope (Olympus U-SPT; Olympus Italy, Rome, Italy). The number of inflammatory cells was expressed as a mean of 10 fields at 100 times magnification.

Nasal lavage was performed with 5 ml of saline, according to standard methods described elsewhere [18]. The fluid was collected to evaluate cytokine levels and stored at  $-20^{\circ}\text{C}$ . The cytokines (IL-4, IL-5, IL-8, and IFN $\gamma$ ) were measured using ELISA (R&D Systems, Minneapolis, Minn., USA). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for each cytokine was precoated onto a microplate. Study samples and standard dilutions of each cytokine were assayed separately in duplicate according to the manufacturer's instructions to the ELISA kit. Standards and samples were pipetted into the wells, and each cytokine was bound by the immobilized antibody. After removing unbound substances, an enzyme-linked polyclonal antibody specific for each cytokine was added to the wells, then the substrate solution was added to develop colored reaction. The reaction was then stopped, and intensity was read at 490 nm with a spectrophotometer (Molecular Devices Corporation, Sunnyvale, Calif., USA). The average net optical density of the standards was plotted, and the amount of each cytokine in each sample was evaluated by interpolation from the standard curve. Results were the means of duplicate samples.

#### Rhinomanometry

Nasal airflow was measured by active anterior rhinomanometry [19] with a computerized instrument (ZAN 100 Rhino; ZAN, Messgeräte, Munich, Germany). Patients wore a tight-fitting facemask, with the mouth closed, and breathed through one nostril. A sensor, placed in the contralateral nostril, recorded data on pre- and post-nasal pressures via airflow and pressure transducers. The signals of transnasal airflow and pressure were amplified, digitized and saved for statistical analysis. Nasal airflow was reported as the sum of the recorded airflow through right and left nostrils (in ml/s) at a pressure difference of 150 Pa across the nasal passage.

#### Spirometry and Methacholine Bronchial Challenge

Spirometry was performed with a computer-assisted spirometer (Pulmolab 435-spiro 235; Morgan Medical Ltd., Gillingham, Kent, UK) according to the European Respiratory Society guidelines [20]. Reference values of the European Community for Steel and Coal were used. If FEV $_1$  was  $\geq 80\%$  of predicted, a methacholine challenge test [21] was carried out in subjects with a normal baseline FEV $_1$ . In the remainder, a reversibility test was performed, with 200  $\mu\text{g}$  salbutamol. Methacholine was administered using a dosimetric computerized apparatus (MEFAR MB3; Markos, Bovezzo, Italy), activated by the inhalator effort. Subjects inhaled progressively increasing doses of methacholine, starting from 30 up to 1,200  $\mu\text{g}$  in 11 steps. The procedure was stopped when FEV $_1$  fell more than 20% compared to baseline. A computerized algorithm provided the provocation dose (PD20) value. If no response was obtained with the maximal cumulative dose of 1,200  $\mu\text{g}/\text{ml}$ , the test was considered negative. The methacholine response was graded as high (PD20 < 200  $\mu\text{g}$ ), moderate (201 < PD20 < 800), low (801 < PD20  $\leq$  1,200) and absent (PD20 > 1,200  $\mu\text{g}$ ).

**Table 1.** Descriptive statistics of the parameters measured

|                                  | Mean  | SD     | Min   | Max   |
|----------------------------------|-------|--------|-------|-------|
| TSS                              | 9.5   | 1.63   | 7.0   | 12.0  |
| Obstruction                      | 2.5   | 0.50   | 2.0   | 3.0   |
| Eosinophils                      | 10.5  | 3.94   | 3.0   | 18.0  |
| Neutrophils                      | 21.3  | 5.91   | 9.0   | 33.0  |
| FEV $_1$ , liters                | 97.9  | 10.75  | 69.0  | 116.0 |
| FVC, liters                      | 97.2  | 8.60   | 80.0  | 118.0 |
| FEF $_{25-75\%}$ , l/s           | 88.3  | 13.28  | 48.0  | 110.0 |
| Methacholine PD20, $\mu\text{g}$ | 171.4 | 259.12 | 0.0   | 918.0 |
| Nasal flow, ml/s                 | 482.5 | 111.42 | 287.0 | 716.0 |
| IL-4, pg/ml                      | 10.2  | 3.08   | 4.8   | 15.3  |
| IL-5, pg/ml                      | 6.5   | 1.73   | 3.7   | 9.4   |
| IL-8, pg/ml                      | 662.2 | 119.78 | 423.0 | 874.0 |
| IFN $\gamma$ , pg/ml             | 1.6   | 0.64   | 0.7   | 2.8   |

TSS = Total symptom score.

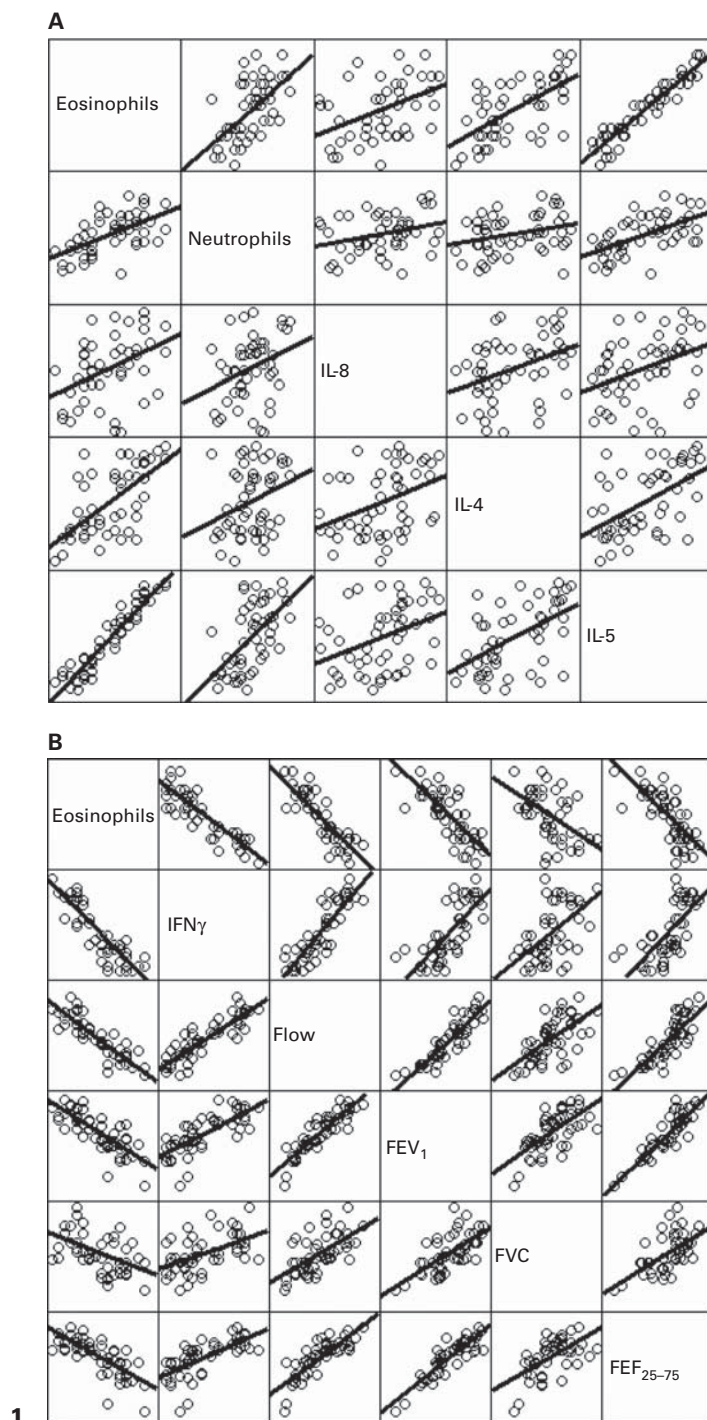
#### Statistical Analysis

To verify the association of functional and clinical measurements, we first used the Pearson correlation coefficient [22]. The Kruskal-Wallis test was used to test for variation in eosinophil and neutrophil numbers among the four methacholine classes [23]. The probability level has been computed using a complete randomization method (permutation or exact test;  $P_{\text{exact}}$ ) or by a Monte Carlo simulation based on 10,000 sampled tables ( $P_{\text{Monte Carlo}}$ ) when computation was not possible [24, 25]. Multiple comparisons of eosinophil and neutrophil numbers between the different methacholine classes were taken into account by correcting the  $\alpha$  level using the Dunn-Šidák significance level correction method:  $\alpha' = 1 - (1 - \alpha)^{1/k}$ , where  $k$  is the number of comparisons [22]; in our case, comparing the effect of the four different methacholine classes, the new significance levels were set to:  $\alpha_{0.05}' = 0.008$ ,  $\alpha_{0.01}' = 0.0017$ ,  $\alpha_{0.001}' = 0.000167$ .

## Results

Fifty consecutive patients were included in the study: all of them were male (age:  $23.7 \pm 4.9$  years). Eight and 7 subjects had single sensitization to *Parietaria* and grasses, respectively, 29 were sensitized to both, 4 were positive to olive and *Parietaria* and 2 to olive and grasses. Only 2 of the subjects had an FEV $_1$  <80% despite the complete absence of perceived bronchial symptoms.

All patients had nasal symptoms during the pollen season, which was confirmed by the presence of high clinical scores and inflammatory cells, as expected (table 1). We performed a series of statistical correlations among the parameters measured. Figure 1 shows the correlations emerging when examining nasal inflammatory cells and cytokines during the pollen season, and table 2 summa-



izes the p values of those correlations. It was found that the number of eosinophils in nasal scrapings highly correlated with all the above-mentioned parameters, including nasal flow and spirometric values. As expected, a significant positive correlation was also found between all

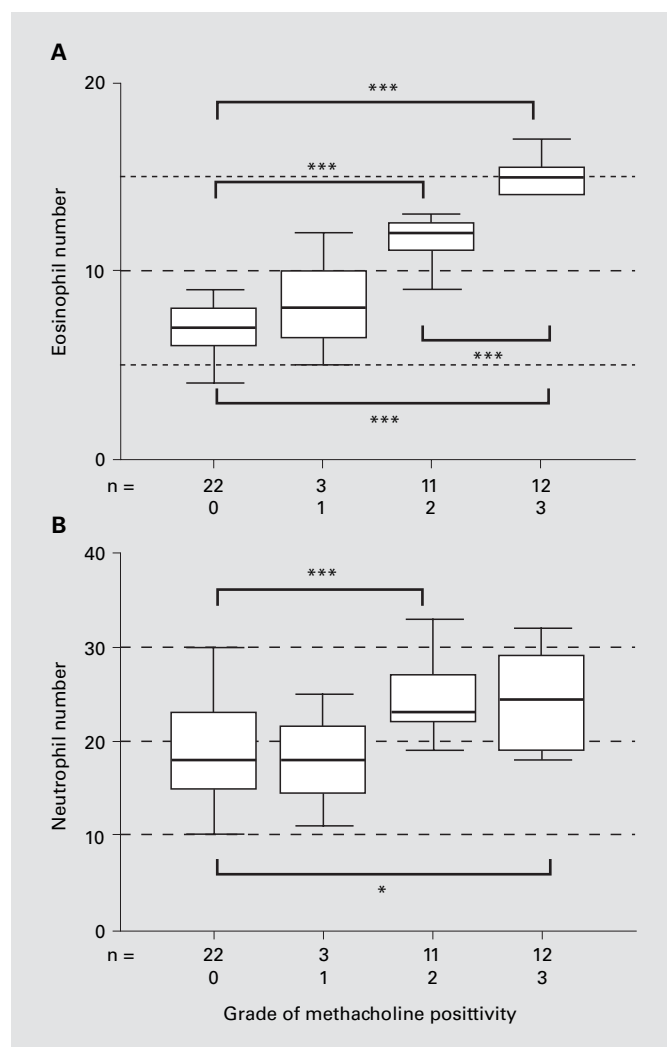


**Fig. 1.** Scatter plot matrices (and regression lines) of the parameters measured against one another. **A** Cells and cytokines. **B** Eosinophils, nasal flow and spirometric values. Each correlation can be read in two ways.

**Fig. 2.** Comparison of eosinophil (**A**) and neutrophil (**B**) numbers among different methacholine classes. Significance levels for the Kruskal-Wallis test were reported in the figure. Significant results (corrected for multiple comparison) of the Mann-Whitney test are also reported:  $*P_{\text{MonteCarlo}} < 0.05$ ;  $**P_{\text{MonteCarlo}} < 0.01$ ;  $***P_{\text{MonteCarlo}} < 0.001$ .

inflammatory cells and all cytokines. Of note, IL-8, IL-4 and neutrophils displayed only a partial correlation with the spirometric parameters ( $FEV_1$ , FVC and  $FEF_{25-75\%}$ ), whereas IL-5, eosinophils and  $IFN\gamma$  correlated with all three. There was also a strong correlation with the objec-

**Table 2.** Correlations among several clinical and inflammatory parameters during the pollen season

|                  | Neutrophils  | IL-8         | IL-4         | IL-5         | IFN $\gamma$  | Nasal flow    | TSS           | FEV <sub>1</sub> | FVC           | FEF <sub>25–75</sub> |
|------------------|--------------|--------------|--------------|--------------|---------------|---------------|---------------|------------------|---------------|----------------------|
| Eosinophils      |              |              |              |              |               |               |               |                  |               |                      |
| r                | <b>0.590</b> | <b>0.439</b> | <b>0.638</b> | <b>0.950</b> | <b>–0.888</b> | <b>–0.854</b> | <b>0.824</b>  | <b>–0.786</b>    | <b>–0.513</b> | <b>–0.746</b>        |
| p                | 0.000        | 0.001        | 0.000        | 0.000        | 0.000         | 0.000         | 0.000         | 0.000            | 0.000         | 0.000                |
| Neutrophils      |              |              |              |              |               |               |               |                  |               |                      |
| r                |              | <b>0.312</b> | <b>0.296</b> | <b>0.592</b> | <b>–0.575</b> | <b>–0.410</b> | <b>0.404</b>  | <b>–0.293</b>    | <b>–0.082</b> | <b>–0.238</b>        |
| p                |              | 0.027        | 0.037        | 0.000        | 0.000         | 0.003         | 0.004         | 0.039            | 0.572         | 0.096                |
| IL-8             |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              | <b>0.382</b> | <b>0.382</b> | <b>–0.309</b> | <b>–0.289</b> | <b>0.355</b>  | <b>–0.263</b>    | <b>–0.145</b> | <b>–0.313</b>        |
| p                |              |              | 0.006        | 0.006        | 0.029         | 0.042         | 0.011         | 0.065            | 0.316         | 0.027                |
| IL-4             |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              |              | <b>0.544</b> | <b>–0.465</b> | <b>–0.441</b> | <b>0.383</b>  | <b>–0.437</b>    | <b>–0.165</b> | <b>–0.507</b>        |
| p                |              |              |              | 0.000        | 0.001         | 0.001         | 0.006         | 0.002            | 0.252         | 0.000                |
| IL-5             |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              |              |              | <b>–0.898</b> | <b>–0.856</b> | <b>0.794</b>  | <b>–0.787</b>    | <b>–0.586</b> | <b>–0.753</b>        |
| p                |              |              |              |              | 0.000         | 0.000         | 0.000         | 0.000            | 0.000         | 0.000                |
| IFN $\gamma$     |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              |              |              |               | <b>0.874</b>  | <b>–0.820</b> | <b>0.756</b>     | <b>0.543</b>  | <b>0.700</b>         |
| p                |              |              |              |              |               | 0.000         | 0.000         | 0.000            | 0.000         | 0.000                |
| Nasal flow       |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              |              |              |               |               | <b>–0.911</b> | <b>0.897</b>     | <b>0.631</b>  | <b>0.828</b>         |
| p                |              |              |              |              |               |               | 0.000         | 0.000            | 0.000         | 0.000                |
| TSS              |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              |              |              |               |               |               | <b>–0.776</b>    | <b>–0.556</b> | <b>–0.750</b>        |
| p                |              |              |              |              |               |               |               | 0.000            | 0.000         | 0.000                |
| FEV <sub>1</sub> |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              |              |              |               |               |               |                  | <b>0.665</b>  | <b>0.883</b>         |
| p                |              |              |              |              |               |               |               |                  | 0.000         | 0.000                |
| FVC              |              |              |              |              |               |               |               |                  |               |                      |
| r                |              |              |              |              |               |               |               |                  |               | <b>0.598</b>         |
| p                |              |              |              |              |               |               |               |                  |               | 0.000                |

TSS = Total symptom score.

tively measured nasal flow and the spirometric indexes. Of the three spirometric values, FVC displayed the weakest correlation with other parameters (table 2). Concerning BHR, subdivided into four classes as described above, there were significant differences ( $\chi^2 = 34.711$ , d.f. = 3,  $P_{\text{Monte Carlo}} < 0.001$ ) in the number of eosinophils in the nasal smear, thus showing a strong linear correlation between methacholine positivity and eosinophils. Despite of an overall significance ( $\chi^2 = 12.564$ , d.f. = 3,  $P_{\text{Monte Carlo}} = 0.003$ ), no comparable linear relationship was found with neutrophils (fig. 2), as the multiple comparisons highlighted that observed differences were most-

ly due to the step between class 0 and classes 2 and 3, whilst no significant differences emerged between the other classes.

## Discussion

The pathogenic mechanisms of AR are quite well known in many aspects. The pathway leading from the allergen-IgE-mast cell interaction to the chronic inflammatory process has been deeply studied in clinical trials involving different models such as the effects of drugs, or

nasal challenges. In general, clinical symptoms were shown to parallel the inflammatory cell infiltration, and the allergic inflammation is overall a Th2-mediated inflammation [18, 26]. Moreover, given the morphofunctional link existing between nose and bronchi, some objective measures of the interaction between the two compartments have been repeatedly provided [9].

Of course, the measurement of cytokines or the morphologic assessment of the bronchial inflammation can be performed only for experimental purposes and cannot be applied to everyday clinical practice. Nevertheless, some clinical tests, such as nasal cell count, spirometry or methacholine bronchial challenge, can be easily performed. Using these simple measures, it was possible to show that a correlation exists among eosinophils, methacholine reactivity, spirometric parameters and nasal symptoms [18, 27, 28].

In the present study in the nose and bronchi, we aimed to obtain a complete and contemporary cross-evaluation of commonly measurable parameters, such as cell count, spirometry, methacholine challenge and rhinomanometry. In order to have a more complete picture, the inflammatory cytokines in nasal lavages were also measured. We chose, for those assessments, patients with pure rhinitis induced by pollens, because the pollinosis model avoids the problems encountered with the variable exposure to perennial allergens, which is difficult to control.

The first observation is that there is a very good correlation between clinical symptoms, inflammatory cells and inflammatory cytokines. Of course this was expected, since numerous studies have previously demonstrated the effects of allergen exposure on nasal inflammation. Nevertheless, in this study, several parameters were assessed at the same time, and it was possible to show that only eosinophils correlate with all the remaining inflammatory parameters. Moreover, we provided a formal demonstration that a clear inverse correlation exists among eosinophils and functional respiratory parameters, supporting one more time the strict link between nose and bronchi. Of note, the entity of the nasal eosinophilic infiltrate correlated well with the degree of methacholine responsiveness in the bronchi. This fact was previously assessed only in a small population of mite-allergic children [29]. Other studies have attempted to correlate different parameters at the same time. In one study [30], various nasal parameters, including eosinophils, were assessed, but only 20 patients were studied. Moreover, those patients had rhinitis and associated asthma, and the only parameter linking upper and lower airways was FEV<sub>1</sub>. In another study [18] in which immunological and

clinical parameters were assessed at the same time, a correlation between eosinophils, symptoms and nasal flow was noted, but the lower airways were not evaluated.

In conclusion, the most important result of the present study is the direct relationship of eosinophil infiltration, cytokines, nasal obstruction (subjectively and objectively measured) and pulmonary function. This confirms the close association between allergic inflammation and nasal airflow impairment, thus supporting a clear structure-function relationship during allergic inflammation. Eosinophils were shown to be the only independent marker of nasal flow, thus confirming their main pathogenic role. More interestingly, nasal eosinophils strictly correlated with spirometric indexes and carefully predicted early bronchial impairment and BHR. The present study provides the first evidence of the relationships of nasal obstruction, eosinophil infiltration, Th2-derived cytokines, nasal and bronchial airflow, and BHR in patients with seasonal allergic rhinitis. Taken together, these data may be considered a supportive proof of the role of eosinophils and Th2 cytokines as a hallmark of nasal obstruction and bronchial involvement.

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