

## Mucosal and systemic inflammatory changes in allergic rhinitis and asthma: a comparison between upper and lower airways

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### Summary

**Background** Local airway inflammation and airway remodelling are considered important in the clinical expression of allergic asthma.

**Objective** The aim of this study was to compare airway inflammation and remodelling in nasal and bronchial mucosa of subjects with allergic rhinitis with or without asthma.

**Methods** Four experimental groups were formed: allergic asthma and rhinitis ( $n = 19$ ); allergic rhinitis, no asthma ( $n = 18$ ); atopic subjects, no asthma, no rhinitis ( $n = 8$ ) and non-allergic healthy control subjects ( $n = 16$ ). Blood samples, nasal and bronchial biopsy specimens were collected during stable disease. Immunohistochemistry was performed for eosinophils (MBP), mast cells (CD117) and vascular endothelium (CD31). Epithelial loss, reticular basement membrane (RBM) thickness and subepithelial vascularity was assessed with a computer-assisted image analysis system.

**Results** In nasal and bronchial mucosa, numbers of eosinophils were significantly higher in rhinitis patients with and without asthma than in asymptomatic atopics ( $P < 0.05$ ) and controls ( $P \leq 0.01$ ). In bronchial mucosa, the RBM was significantly thickened in rhinitis patients with and without asthma compared to asymptomatic atopics ( $P < 0.05$ ) and controls ( $P < 0.01$ ), while in nasal mucosa no differences were seen. Patients with asthma and rhinitis had increased numbers of blood eosinophils ( $P = 0.05$ ) and skin test reactivity ( $P = 0.01$ ) compared to patients with rhinitis only. No significant differences could be found between the investigated groups with respect to serum IL-5 and eotaxin levels, the number of mucosal mast cells and the degree of epithelial loss and subepithelial vascularity. Epithelial desquamation was significantly increased in the bronchial mucosa compared to nasal mucosa, not only in asthmatics ( $P < 0.001$ ), but also in atopics without asthma and rhinitis ( $P = 0.02$ ).

**Conclusions** This study shows that allergic inflammation, increased basement membrane thickness and epithelial desquamation are present in the lower airways of atopic subjects, even before the onset of clinical symptoms. Despite the presence of inflammatory cells, no structural changes could be assessed in nasal mucosa of allergic patients.

**Keywords** airway remodelling, allergic rhinitis, asthma, clinical expression, mucosal inflammation

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### Introduction

Allergic rhinitis and asthma are two entities of the atopic syndrome that commonly occur together [1]. The discrimination between allergic rhinitis and asthma can be difficult, as symptom perception is widely variable; lung function can be normal in mild asthmatics and, although bronchial hyper-responsiveness (BHR) is a constant feature of asthma, it is also frequently present in allergic rhinitis [2].

Chronic airway inflammation has been considered an important hallmark in both asthma and rhinitis. Allergic rhinitis and asthma are characterized by a similar inflammatory process in

which mast cells and eosinophils are important effector cells [3, 4]. Although there is no apparent clinical manifestation of lower airway inflammation, inflammatory cells and mediators have been found in the lower airways of allergic rhinitis patients without asthma, as well [5, 6]. Similarly, eosinophil infiltration has been demonstrated in the upper airways of asthmatic patients, even in the absence of rhinitis [7]. This illustrates that the relationship between cellular aspects of inflammation and clinical expression is not straightforward.

Airway remodelling is mentioned as one of the factors accounting for airflow obstruction and bronchial hyper-responsiveness in allergic asthma. Structural changes in the bronchial wall, typical for asthma, are loss of epithelial lining [8, 9], reticular basement membrane (RBM) thickening [8, 10] and increased vascularity in the subepithelial layer [11, 12].

Few studies have investigated upper and lower airways simultaneously in atopic patients. One study compared nasal and

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bronchial inflammation in asthmatic patients with perennial rhinitis and found more eosinophilia and structural changes in bronchial than in nasal mucosa [3]. Non-asthmatic allergic subjects were not included in this study, however. We have previously demonstrated that generalized airway inflammation is present in non-asthmatic allergic rhinitis patients after segmental bronchial provocation (SBP) and after nasal provocation (NP) [5, 13]. Moreover, we detected increased expression of serum-IL-5 and numbers of circulating eosinophils in allergic rhinitis patients after SBP, which suggests that a systemic allergic response is triggered [14].

To shed more light on the relationship between clinical parameters and inflammatory changes, local as well as systemic, we designed a study in which blood samples and nasal and bronchial mucosal biopsy specimens were taken from atopic patients with various expression of allergic airways' disease. The aim of the study was to compare mucosal allergic inflammation, features of airway remodelling and systemic inflammatory markers in three groups of allergic patients: those with perennial allergic rhinitis and asthma; those with perennial allergic rhinitis, no asthma; and those with just atopy, without any respiratory symptoms. A group of non-allergic healthy subjects served as controls. We hypothesized that generalized airway inflammation, airway remodelling and elevated levels of inflammatory mediators in the circulation would be features of atopic disease, regardless of clinical expression.

Mast cells (CD117) and eosinophils (MBP) were chosen as markers of mucosal allergic inflammation. The degree of epithelial loss, RBM thickness and subepithelial vascularity were taken as features of airway remodelling. Blood eosinophils, serum-IL-5 and serum-eotaxin served as markers of systemic inflammation.

## Materials and methods

### *Subject groups and study design*

Inclusion of subjects started in June 1998 and was completed in February 2000. Patients were selected on the basis of persistent perennial nasal and/or bronchial symptomatology. Four experimental groups were formed: allergic rhinitis and asthma ( $n = 19$ ); allergic rhinitis, no asthma ( $n = 18$ ); atopic subjects, no asthma, no rhinitis ( $n = 8$ ); and non-allergic healthy control subjects ( $n = 16$ ). All patients were allergic to house dust mite. Allergy was assessed by skin prick test. All subjects were tested for a panel of 14 common inhalant allergens (Vivodiagnost, ALK Benelux BV, Groningen, the Netherlands). A skin prick test was considered positive when a weal diameter  $> 3$  mm was recorded after 15 min. Other allergens, such as dander or pollen, were allowed. Controls had a negative skin prick test. Asthmatic patients fulfilled the criteria for asthma, according to GINA guidelines [15]. Diagnosis of allergic rhinitis was based on criteria recently published in an EAACI consensus statement [16]. The atopy score was determined in a semiquantitative way [17]: 0 = no different from saline control, + = 1–2 mm larger than control, ++ = 3–5 mm larger than control, +++ = 6–8 mm larger than control, ++++  $\geq 8$  mm larger than control. For each subject, a composite score was obtained, adding the results (number of +) of all 14 skin prick test reactions together.

All subjects were non-smokers. Treatment with short-acting bronchodilators was allowed up to 8 h, and long-acting bronchodilators and antihistamines up to 48 h prior to investigation. Treatment with sodium cromoglycate, astemizole, inhaled or intranasal corticosteroids was stopped at least 6 weeks before entering the study. None of the study subjects had used oral or systemic corticosteroids 6 months prior to the study.

All patients and control subjects were in stable clinical condition: no subject had experienced an infection of the respiratory tract, an acute asthmatic attack or hospital admission 6 weeks prior to investigation. All participants gave informed written consent to the study, which was approved by the medical ethics committee of the Erasmus University Medical Center Rotterdam, the Netherlands.

### *Clinical parameters*

Signs and symptoms were scored at the beginning of the visit on a 10-cm visual analogue scale (VAS). Symptoms were divided into nasal and ocular complaints (rhinorrhoea, watery eyes, nasal itching, sneezing and nasal blockage) and pulmonary complaints (wheezing, coughing, shortness of breath and exercise intolerance) [5, 6].

Upper and lower airways obstruction were determined with peak nasal inspiratory flow (PNIF) and forced expiratory volume in 1 s (FEV1) measurements. PNIF was measured by a Youlten peak nasal inspiratory flow meter (Armstrong Industries, Inc., Northbrook, IL, USA). FEV1 was determined by standard spirometry. All study subjects had a FEV1  $> 60\%$  of predicted. The provocative concentration of methacholine causing a 20% decrease in FEV1 (PC20 methacholine) was  $\leq 8$  mg/mL in asthmatic patients. All non-asthmatic patients had values  $> 8$  mg/mL. Methacholine was administered according to a standardized tidal breathing method [13].

### *Blood samples*

Blood sampling and collection of bronchial and nasal biopsy specimens were performed on the same day. The total number of blood eosinophils was determined by haemocytometric differential cell count (Sysmex NE 8000). IL-5 and eotaxin were measured in serum with commercially available enzyme-linked immunosorbent assay (ELISA) kits, following the instructions of the manufacturer (Cytoscreen™, BioSource International Inc., Camarillo, CA, USA). The lowest detection level of IL-5 was 11.7 pg/mL. The minimal detectable dose of eotaxin was 7.8 pg/mL. No cross-reactivity was found with other cytokines.

### *Bronchial biopsies*

All bronchial biopsies were taken by the same pulmonary physician (SEO). After intramuscular pre-medication with atropine dinitrate (0.5 mg), oropharyngeal anaesthesia was accomplished with topical xylocaine spray 1%. Next, the vocal cords, trachea and bronchial tree were anaesthetized with oxybuprocaine. The fiberoptic bronchoscope (BF, type P20 D; Olympus, Tokyo, Japan) was introduced into the airway via the oral route and mucosal biopsies were taken from the segmental carinae of the left and right lung. Bronchial biopsies were embedded in Tissue-Tek II Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA, USA), frozen and stored at  $-150^{\circ}\text{C}$ .

### Nasal biopsies

All biopsy specimens of the nasal mucosa were taken by the same investigator (GJB). First, local anaesthesia was induced by placing a cotton-wool carrier with 50–100 mg of cocaine and three drops of epinephrine (1 : 1000) under the inferior turbinate, without touching the biopsy site. Secondly, a mucosal biopsy sample was obtained from the lower edge of the inferior turbinate about 2 cm posterior to the edge, using a Gerritsma forceps with a cup diameter of 2.5 mm. The nasal biopsies were embedded in Tissue-Tek II OCT compound, frozen and stored at  $-150^{\circ}\text{C}$ .

### Monoclonal antibodies

The monoclonal antibodies (mAbs) used in this study were antimajor basic protein (MBP) antibody (IgG1, 0.2  $\mu\text{g/mL}$ , clone nr. BMK13, Sanbio, Uden, the Netherlands) for identifying eosinophils, anti-CD117 antibody (IgG1, 1  $\mu\text{g/mL}$ , clone nr. YB5.B8, Pharmingen) for mast cells and anti-human endothelium (CD31) antibody (IgG1, 1  $\mu\text{g/mL}$ , clone nr. EN4, Sanbio, the Netherlands).

### Immunohistochemical staining

Each tissue specimen was cut into serial 6- $\mu\text{m}$ -thick sections. The mAb stainings were developed with the supersensitive immunoalkaline phosphatase (ss-AP) method as previously described [13]. Isotype-specific control staining was done with mouse IgG1 antibody to *Aspergillus niger* glucose oxidase (Dako cat. no. X 0931) at the same protein concentration as the specific antibody.

### Microscopic assessment of immunohistochemical staining

Biopsies were coded and two sections, at least 120  $\mu\text{m}$  apart, were counted blind for each antibody as has been described previously [13]. Bronchial and nasal sections were divided into epithelium and subepithelium (area 100  $\mu\text{m}$  deep into the lamina propria along the length of the epithelial basement membrane). Positively stained inflammatory cells localized in epithelium and subepithelium were counted along the basement membrane, which had to be undamaged for a length of at least 1 mm before being accepted for evaluation. Cell numbers in epithelium were expressed as positively stained cells per mm basement membrane (BM); subepithelial cell numbers were expressed as the number of cells/ $\text{mm}^2$ , using an Axioskop 20 microscope (Zeiss, Jena, Germany) with an eyepiece graticule at a magnification of  $\times 200$ .

### Quantification of remodelling

Nasal and bronchial sections were recorded using a computer assisted image analysis system (Leica, Rijswijk, the Netherlands). This method is based on densitometric differences between positively stained tissue and background after setting thresholds for colour, saturation and intensity [5]. If the total area studied was less than 100 000  $\mu\text{m}^2$  for a given subject, this subject was excluded from further analysis.

### Reticular BM thickness

Thickness of the reticular BM was interactively measured on  $40 \times 10$  magnified images at regular intervals following recommendations described recently [18].

### Epithelial shedding

At the same images, the reticular BM occupancy with epithelium (at least one cell layer), plotted as ratio of occupied membrane length divided by the total membrane length, was determined for the investigated groups at  $40 \times 10$  magnification.

### Subepithelial vascularity

Vessels with a distinct lumen staining positive for CD31 were counted on  $20 \times 10$  magnified images in an area 100  $\mu\text{m}$  deep in the lamina propria along the length of the epithelial BM.

### Statistical analysis

Data are presented as medians  $\pm$  range and mean  $\pm$  SEM as indicated. Statistical analysis was performed using Wilcoxon's *W*-test for intra group analysis, and Kruskal–Wallis and Mann–Whitney *U*-test for inter group analysis. Data were correlated by the Spearman rank test. A *P*-level of  $< 0.05$  was considered significant for intra-subgroup analyses. When more than two samples were compared, a correction for multiple testing of data was made by increasing the level of significance ( $P \leq 0.02$ ).

## Results

### Subject characteristics

The subject groups were comparable for sex, age, vital capacity (VC) and FEV1 (Table 1). By definition, asthmatic patients had a significantly decreased PC20 (methacholine), lower FEV1/VC ratio and more pulmonary symptoms compared to non-asthmatic subjects. Also, rhinitis patients had more nasal symptoms than non-rhinitic subjects. No difference in PNIF and total nasal symptom score was detectable between patients with and without asthma. By definition, control subjects had an atopy score of zero. Patients with asthma and rhinitis had a higher atopy score than patients with rhinitis only ( $P = 0.01$ ). A positive family history for allergy was found more frequently in rhinitis patients with and without asthma than in control subjects.

### Immunostaining of mucosal biopsies

**General description** We collected one nasal and several bronchial mucosa biopsy specimens per patient. One hundred and sixteen out of 122 specimens reached criteria for evaluation. The mean length of BM that could be evaluated was  $5.3 \text{ mm} \pm 0.20$  (SEM) in bronchial mucosa and  $6.6 \text{ mm} \pm 0.30$  in nasal mucosa. Epithelium and/or subepithelium could not be evaluated in five bronchial samples and one nasal sample. These samples were excluded from the study.

### Nasal specimens

**Inflammatory cells** The results are outlined in Table 2. In nasal epithelium, the numbers of eosinophils were significantly higher in patients with asthma and rhinitis ( $P = 0.05$ ) and rhinitis only ( $P = 0.05$ ) compared to control subjects. There were more mast cells present in patients with asthma and rhinitis than in asymptomatic atopics and control subjects ( $P = 0.02$ ).

**Table 1.** Patient characteristics

|                             | HC            | A <sup>-</sup> R <sup>-</sup> | A <sup>-</sup> R <sup>+</sup> | A <sup>+</sup> R <sup>+</sup> | P-value† |
|-----------------------------|---------------|-------------------------------|-------------------------------|-------------------------------|----------|
| Number                      | 16            | 8                             | 18                            | 19                            |          |
| Sex                         |               |                               |                               |                               |          |
| Male                        | 8             | 3                             | 9                             | 6                             | NS       |
| Female                      | 8             | 5                             | 9                             | 13                            | NS       |
| Age (years)                 | 23.5 (18–46)  | 23.5 (23–31)                  | 29 (18–59)                    | 27 (19–57)                    | NS       |
| Atopy score (N+)            | 0             | 3 (2–9)*                      | 8 (3–20)*                     | 14 (7–27)*                    | < 0.001  |
| Positive family history (%) | 37.5          | 37.5                          | 81*                           | 79*                           | 0.008    |
| PC20 (mg/mL)                | 40 (32.4–40)  | 40 (16.9–40)                  | 40 (9.3–40)                   | 1.9 (0.2–6.1)*                | < 0.001  |
| VC (%)                      | 104 (73–123)  | 100 (85–122)                  | 100 (86–120)                  | 108 (88–126)                  | NS       |
| FEV1 (%)                    | 106 (75–118)  | 106 (83–122)                  | 103 (78–129)                  | 101 (62–128)                  | NS       |
| FEV1/VC (%)                 | 101 (89–114)  | 102 (94–110)                  | 102 (92–118)                  | 94 (70–104)*                  | 0.004    |
| PNIF (L/min)                | 232 (100–320) | 187 (155–230)                 | 180 (120–290)                 | 180 (80–250)                  | NS       |
| VAS nose (mm)               | 9 (0–38)      | 19 (0–46)                     | 53 (5–229)*                   | 74 (10–371)*                  | < 0.001  |
| VAS lung (mm)               | 0 (0–31)      | 9 (0–41)                      | 8 (0–46)                      | 33 (0–259)*                   | 0.006    |

HC, healthy control subjects; A<sup>-</sup>R<sup>-</sup>, atopic subjects, no asthma, no rhinitis; A<sup>-</sup>R<sup>+</sup>, allergic rhinitis, no asthma; A<sup>+</sup>R<sup>+</sup>, allergic asthma and rhinitis; VC, vital capacity; PNIF, peak nasal inspiratory flow; VAS, visual analogue scale. Data are expressed as medians and range. \**P* < 0.05 compared to HC group, Mann–Whitney *U*-test. †A Kruskal–Wallis statistical analysis was performed to compare the groups. NS = *P* > 0.02.

**Table 2.** Inflammatory cells mucosa

| Organ | Cell type          | Level  | HC           | A <sup>-</sup> R <sup>-</sup> | A <sup>-</sup> R <sup>+</sup> | A <sup>+</sup> R <sup>+</sup> | P-value† |
|-------|--------------------|--------|--------------|-------------------------------|-------------------------------|-------------------------------|----------|
| Nose  | MBP <sup>+</sup>   | Epi    | 0 (0–1)      | 0 (0–1)                       | 0 (0–12)                      | 0 (0–11)                      | NS       |
|       |                    | Subepi | 7 (0–30)     | 1 (0–29)                      | 16 (0–181)*                   | 38 (2–214)*                   | 0.002    |
|       | CD117 <sup>+</sup> | Epi    | 1 (0–3)      | 1 (0–1)                       | 0 (0–8)                       | 2 (0–20)                      | NS       |
|       |                    | Subepi | 103 (43–188) | 96 (55–162)                   | 78 (0–167)                    | 108 (11–193)                  | NS       |
| Lung  | MBP <sup>+</sup>   | Epi    | 0 (0–1)      | 0 (0–1)                       | 0 (0–5)*                      | 0 (0–5)*                      |          |
|       |                    | Subepi | 3 (0–13)     | 8 (0–25)                      | 41 (0–115)*                   | 42 (2–158)*                   | < 0.001  |
|       | CD117 <sup>+</sup> | Epi    | 0            | 0 (0–1)                       | 0 (0–4)                       | 0 (0–6)                       | NS       |
|       |                    | Subepi | 32 (5–110)   | 20 (8–125)                    | 58 (0–143)                    | 45 (0–195)                    | NS       |

HC, healthy control subjects; A<sup>-</sup>R<sup>-</sup>, atopic subjects, no asthma, no rhinitis; A<sup>-</sup>R<sup>+</sup>, allergic rhinitis, no asthma; A<sup>+</sup>R<sup>+</sup>, allergic asthma and rhinitis; VC, vital capacity; PNIF, peak nasal inspiratory flow; VAS, visual analogue scale. Data are expressed as medians and range. \**P* < 0.05 compared to HC group, Mann–Whitney *U*-test. †A Kruskal–Wallis statistical analysis was performed to compare the groups. NS = *P* > 0.02.

In subepithelium, we found significantly more eosinophils in patients with asthma and rhinitis compared to asymptomatic atopics (*P* = 0.002) and control subjects (*P* < 0.001). Patients with rhinitis only had higher numbers of eosinophils than asymptomatic atopics (*P* = 0.03) and controls (*P* = 0.05).

No difference could be detected in the number of eosinophils and mast cells between rhinitis patients with and without asthma.

**Structural changes** The results are outlined in Table 3. Three patients had epithelial loss of more than 50% in the nasal mucosa (one control, two rhinitis subjects), whereas in most subjects the epithelium was still completely intact. Between-group differences could not be detected.

The RBM in the nasal mucosa was similar in patient and control groups with a median value between 7.9 and 8.6 µm.

The number of CD31<sup>+</sup> vessels in the nasal subepithelium tended to be higher in atopic patients, although the difference was not significant.

#### Bronchial specimens

**Inflammatory cells** The results are outlined in Table 2. In bronchial epithelium, numbers of eosinophils were significantly

higher in patients with asthma and rhinitis compared to asymptomatic atopics (*P* = 0.02) and controls (*P* = 0.002). Patients with rhinitis only also had increased numbers of eosinophils in the bronchial epithelium compared to asymptomatic atopics (*P* = 0.03) and controls (*P* = 0.01). No difference was found between the rhinitis patients with and without asthma. In atopic subjects, few mast cells were found in the epithelium, while virtually no mast cells could be detected in the epithelium of controls.

In subepithelium, we found a significantly higher number of eosinophils in rhinitis patients with (*P* < 0.001) and without asthma (*P* < 0.001) than in control subjects. Also, in asymptomatic atopics, there was a strong tendency towards an elevated number of eosinophils compared to controls (*P* = 0.06). The numbers of eosinophils and mast cells were not different between rhinitis patients with and without asthma.

**Structural changes** The results are outlined in Table 3. Even in control subjects, some degree of epithelial shedding was present in the lower airways. Epithelial loss was increased in rhinitis patients with and without asthma compared to controls (*P* = 0.03). No difference could be detected between the rhinitis patients with and without asthma.

The RBM in the bronchial mucosa was thicker in patients with asthma and rhinitis than in asymptomatic atopics ( $P=0.007$ ) and controls ( $P<0.001$ ). Patients with rhinitis only had a thicker RBM than asymptomatic atopics ( $P=0.04$ ) and controls ( $P=0.004$ ). Again, no difference was found between rhinitis patients with and without asthma.

The number of CD31<sup>+</sup> vessels in the bronchial subepithelium was not significantly different between the investigated groups.

#### Differences between nasal and bronchial mucosa

**Inflammatory cells** The number of eosinophils was not significantly different between nasal and bronchial epithelium, as well as subepithelium among the investigated subgroups

(Fig. 1). Mast cells, however, were significantly more abundant in nasal epithelium and subepithelium compared to the corresponding areas in the bronchi for healthy controls ( $P<0.01$ ) and patients with asthma and rhinitis ( $P<0.01$ ), but not for the non-asthmatic atopic patients.

**Structural changes** In healthy control subjects, the RBM was significantly thicker in the nasal mucosa compared to the bronchial mucosa ( $P=0.03$ ). In the allergic subgroups, the RBM thickness was similar in nasal and bronchial mucosa (Fig. 2). The epithelial loss was significantly increased in bronchial mucosa of the allergic subgroups, but not in healthy controls subjects (Fig. 3).

**Table 3.** Airway remodelling

| Organ                                     | HC             | A <sup>-</sup> R <sup>-</sup> | A <sup>-</sup> R <sup>+</sup> | A <sup>+</sup> R <sup>+</sup> | P-value† |
|-------------------------------------------|----------------|-------------------------------|-------------------------------|-------------------------------|----------|
| <b>Nose</b>                               |                |                               |                               |                               |          |
| Intact epithelium (%)                     | 94 (48–100)    | 91 (78–98)                    | 85 (32–100)                   | 84 (65–100)                   | NS       |
| RBM thickness (µm)                        | 8.6 (5.7–10.5) | 8.2 (5.8–8.3)                 | 8.6 (5.7–12.9)                | 8.4 (6.1–16.4)                | NS       |
| CD31 <sup>+</sup> vessels/mm <sup>2</sup> | 97 (28–268)    | 112 (66–272)                  | 131 (0–202)                   | 136 (13–271)                  | NS       |
| <b>Lung</b>                               |                |                               |                               |                               |          |
| Intact epithelium (%)                     | 84 (45–97)     | 74 (54–89)                    | 75 (29–86)                    | 52 (15–95)                    | NS       |
| RBM thickness (µm)                        | 7.1 (5.2–8.5)  | 7.3 (5.6–8.3)                 | 8.4 (5.9–11.0)*               | 9.2 (6.6–12.6)*               | < 0.001  |
| CD31 <sup>+</sup> vessels/mm <sup>2</sup> | 50 (6–88)      | 95 (22–120)                   | 48 (2–213)                    | 83 (26–182)                   | NS       |

HC, healthy control subjects; A<sup>-</sup>R<sup>-</sup>, atopic subjects, no asthma, no rhinitis; A<sup>-</sup>R<sup>+</sup>, allergic rhinitis, no asthma; A<sup>+</sup>R<sup>+</sup>, allergic asthma and rhinitis; VC, vital capacity; PNIF, peak nasal inspiratory flow; VAS, visual analogue scale; RBM, reticular basement membrane. Data are expressed as medians and range. \* $P<0.05$  compared to HC group, Mann-Whitney *U*-test. †A Kruskal-Wallis statistical analysis was performed to compare the groups. NS =  $P>0.02$ .

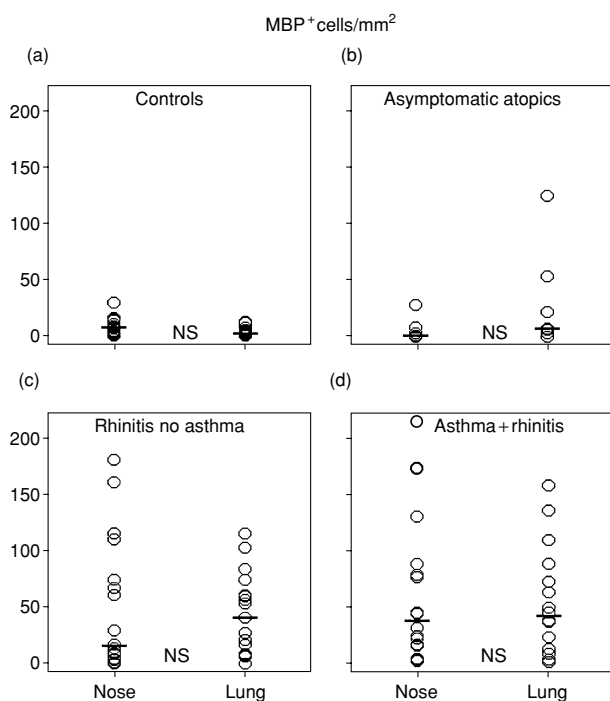


**Fig. 1.** Numbers of eosinophils (major basic protein<sup>+</sup> cells/mm<sup>2</sup>) in nasal and bronchial subepithelial space of (a) healthy control subjects, (b) atopic subjects, no asthma, no rhinitis, (c) allergic rhinitis, no asthma, (d) allergic asthma and rhinitis. Horizontal bars represent medians and open circles represent individual values. Statistical analysis by Wilcoxon's *W*-test. NS =  $P>0.05$ .

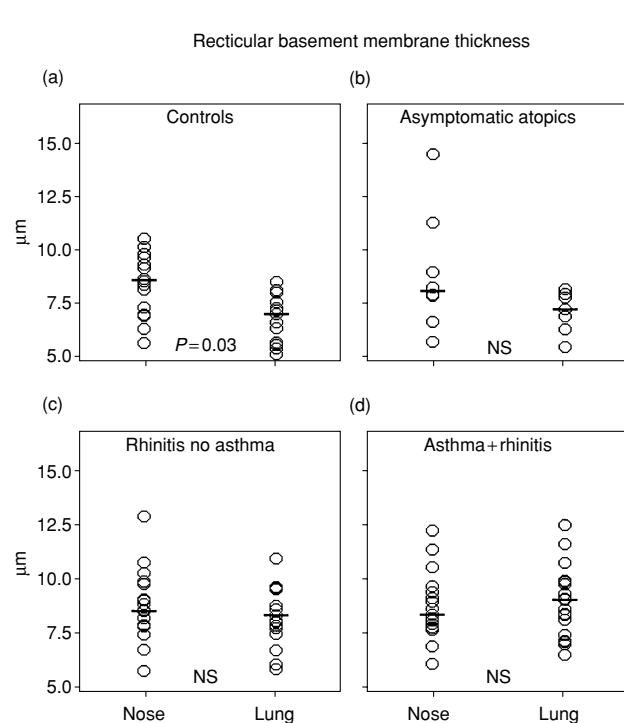


**Fig. 2.** Reticular BM thickness (in µm) in nasal and bronchial biopsies of (a) healthy control subjects, (b) atopic subjects, no asthma, no rhinitis, (c) allergic rhinitis, no asthma, (d) allergic asthma and rhinitis. Horizontal bars represent medians and open circles represent individual values. Statistical analysis by Wilcoxon's *W*-test. NS =  $P>0.05$ .

**Table 4.** Systemic inflammatory mediators

|                                 | HC               | A <sup>-</sup> R <sup>-</sup> | A <sup>-</sup> R <sup>+</sup> | A <sup>+</sup> R <sup>+</sup> | P-value† |
|---------------------------------|------------------|-------------------------------|-------------------------------|-------------------------------|----------|
| Eosinophils ( $\times 10^9/L$ ) | 0.11 (0.03–0.18) | 0.18 (0.08–0.39)              | 0.17 (0.09–0.37)*             | 0.25 (0.02–0.78)*             | < 0.001  |
| IL-5 (pg/mL)                    | 0 (0–46)         | 19 (0–396)                    | 14 (0–213)                    | 16 (0–368)                    | NS       |
| Eotaxin (pg/mL)                 | 48 (33–106)      | 43 (18–290)                   | 61 (13–107)                   | 41 (16–97)                    | NS       |

HC, healthy control subjects; A<sup>-</sup>R<sup>-</sup>, atopic subjects, no asthma, no rhinitis; A<sup>-</sup>R<sup>+</sup>, allergic rhinitis, no asthma; A<sup>+</sup>R<sup>+</sup>, allergic asthma and rhinitis; VC, vital capacity; PNIF, peak nasal inspiratory flow; VAS, visual analogue scale. Data are expressed as medians and range. \* $P < 0.05$  compared to HC group, Mann–Whitney *U*-test. †A Kruskal–Wallis statistical analysis was performed to compare the groups. NS =  $P > 0.02$ .

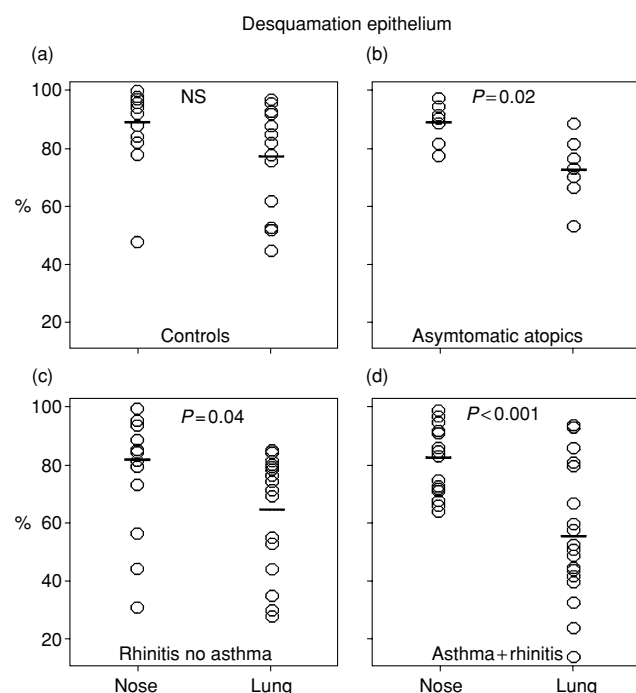


**Fig. 3.** Results of epithelial desquamation (percentage of reticular BM covered by intact epithelium) in nasal and bronchial biopsies of (a) healthy control subjects, (b) atopic subjects, no asthma, no rhinitis, (c) allergic rhinitis, no asthma, (d) allergic asthma and rhinitis. Horizontal bars represent medians and open circles represent individual values. Statistical analysis by Wilcoxon's *W*-test. NS =  $P > 0.05$ .

The number of CD31<sup>+</sup> vessels was higher in nasal mucosa than in bronchial mucosa in all investigated groups ( $P < 0.05$ ), except in asymptomatic atopics.

#### Inflammatory markers blood

Table 4 shows the results of the haemocytometric measurement of peripheral blood eosinophils. Rhinitis patients with and without asthma had increased numbers of blood eosinophils compared to controls ( $P < 0.001$ ,  $P = 0.001$ , respectively). The number of blood eosinophils was higher in rhinitis patients with asthma than without asthma ( $P = 0.05$ ). No differences could be found between the groups with respect to the expression of IL-5 and eotaxin in the serum (Table 4).

#### Correlations between inflammatory markers, airway function and symptomatology

In methacholine-responsive subjects ( $PC_{20} < 38$  mg/mL;  $n = 31$ ),  $PC_{20}$  correlated with atopy score ( $r = -0.40$ ,

$P = 0.018$ ). The number of eosinophils in peripheral blood of all allergic subjects ( $n = 45$ ) correlated with the FEV1/VC ratio ( $r = -0.40$ ,  $P = 0.005$ ) and total nasal symptom score ( $r = 0.38$ ,  $P = 0.008$ ). In allergic subjects, the atopy score correlated with eosinophils in nasal ( $r = 0.46$ ,  $P = 0.001$ ) and bronchial sub-epithelium ( $r = 0.41$ ,  $P = 0.005$ ). No association was found between markers of airway remodelling and the number of eosinophils and mast cells.

#### Discussion

In this study, we demonstrated similar inflammatory changes in upper and lower airways of allergic rhinitis patients with and without asthma. Thickening of the reticular BM and increased epithelial desquamation were present in the lower airways of allergic patients, even in the absence of clinical symptoms. No evidence was found for airway remodelling in the upper airways of allergic patients with rhinitis.

Our observation that inflammation extends to the lower airways in patients with perennial allergic rhinitis is in agreement with previous findings. Djukanovic et al. showed increased numbers of eosinophils in bronchial tissue of allergic rhinitis patients without asthma [19]. Gaga et al. demonstrated allergic inflammation in nasal mucosa of non-allergic asthmatic patients without rhinitis [7]. It should be noted, however, that these studies were limited by their small sample size and that they did not represent the total spectrum of allergic disease. Our study is novel in that we have studied clinical as well as subclinical atopic patients, which is important with respect to the natural development of allergic airway disease. In this context, it would also have been interesting to look at atopic patients with asthma but no rhinitis. However, out of 98 patients with asthma who were screened for the study, only three patients fulfilled the criteria for this group. These findings suggest that the prevalence of this phenotype is low in our population. Togias, in a recent epidemiological review, also reports very small numbers for this particular subset of patients [20].

In our study, we found that nasal allergic inflammation was generally related to clinical expression of allergic rhinitis and tended to be more severe in rhinitis patients with concomitant asthma. Albeit, that no correlation could be found between the number of eosinophils and the severity of nasal symptoms. In the bronchial mucosa, eosinophilia was detected in all atopic subjects irrespective of the clinical manifestation of asthma and/or rhinitis. Moreover, the numbers of eosinophils were comparable in rhinitis patients with and without asthma. Nasal eosinophilia was related to bronchial eosinophilia in atopic subjects and eosinophil numbers were in the same range. This is in

contrast with earlier findings of Chanez, who demonstrated increased eosinophil numbers in the bronchial mucosa compared to nasal mucosa [3]. Differences in patient selection, staining techniques and methods of tissue sampling may account for this. We cannot rule out that the difference in inflammatory infiltrate with non-asthmatic subjects could have been more evident when severe cases would have been included in the study, as has been demonstrated by Louis [21]. Our asthmatic patients had only mild disease, according to the GINA guidelines. However, our ethical committee did not allow us to include more severe asthmatic subjects.

Apparently, the presence of eosinophils in the airway mucosa is integrate part of the atopic constitution. The quantity and activity of these cells at any given point in time may determine the clinical manifestation of allergic airway disease be it just asthma or rhinitis or a combination of the two. Triggers may come from natural exposure to allergens or from allergen provocations in an experimental setting. Both have shown to result in increased numbers of eosinophils and mast cells in the nasal subepithelium [5, 22, 23]. It should be noted, that changes in eosinophils and mast cells were related to nasal symptoms in the acute stage of allergic inflammation only [24]. In contrast, our subjects were in chronic, stable phase of disease.

BHR and chronic inflammation are considered important features of allergic asthma. Although several studies have pointed towards the relationship between bronchial inflammation and BHR [6, 25], others failed to detect a relationship [26–28]. In our study, we could not find such an association. In fact, the presence of bronchial inflammation in non-asthmatic atopic patients, even in the absence of any airway symptoms at all, challenges the direct relationship between the presence of inflammatory cells, in particular eosinophils and BHR.

Eosinophil and mast cell degranulation may be relevant in the distinction between the chronic and the acute phase of disease [29]. We also analysed the presence of MBP – irrespective of it being cell bound – in the mucosa of the airways by means of image analysis [30] (data not shown). The results of this analysis were exactly in line with the results of the cellular counts and added no extra information to our earlier findings. Also, additional double-staining of mast cells with chymase and tryptase did not show any difference between the experimental groups (data not shown). We suggest that inflammatory cells are in an 'active' mode during acute inflammation and become 'quiescent' during chronic, stable phase of disease. Allergen provocation studies in nose and lung support this hypothesis. These studies demonstrated persistent inflammation up to 2 weeks after allergen provocation long after symptoms had disappeared [22, 31]. The presence of inflammatory cells *per se* in mucosal tissue may therefore only be footprints of previous immunologic activity.

Increased loss of bronchial epithelium has been demonstrated to be present in asthmatic patients compared to non-asthmatic control subjects [8]. Despite a tendency towards more epithelial shedding in bronchial mucosa of asthmatic subjects, we could not find a clear relationship with clinical manifestations. In our study, bronchial epithelial desquamation is even present before the onset of clinical symptoms. Although several authors reported that the degree of epithelial shedding was associated with BHR [8, 9], others could not find such a relationship. Recently, the role of epithelial shedding in asthma has been questioned by Ordonez et al. who suggested that the loss of

bronchial epithelium may just be an artifact of tissue sampling [32]. This does not explain, however, why we do not see epithelial loss in the upper airways. Despite one single publication describing epithelial loss in patients with perennial rhinitis [33], usually no epithelial desquamation is seen [3, 34]. Chanez et al. using the same biopsy technique in the upper as in the lower airways, found no significant difference in nasal epithelial shedding between rhinitics and controls [3]. Therefore, tissue sampling is not the only explanation for increased epithelial shedding in the lower airways. Presumably, disruption of intraepithelial adhesive mechanisms may play a role in increased epithelial fragility [9, 35].

Thickening of the bronchial reticular BM due to subepithelial collagen deposition is considered as another important feature of allergic asthma. In our study, we were able to detect a weak correlation between thickening of the BM and PC20. This is in agreement with the finding that the degree of BM thickening was related to disease severity in asthmatics [10]. On the other hand, increased BM thickness has been found in recently diagnosed mild asthmatic disease [36], as well as in non-asthmatic allergic subjects [37].

It seems unlikely that reticular BM thickening is important in the pathophysiology of allergic rhinitis. We could not find any difference in reticular BM thickening between rhinitis patients and healthy controls. However, the observation that the reticular BM in the nose is usually as thick as in the bronchi of asthmatic patients, raises the question whether we are not looking at the end stage of a remodelling process. It may very well be that the nose, through its function as filter and air-conditioner of the airways, is exposed to higher concentrations of toxic and microbiological agents compared to the lower airways. An increase in BM thickening in the nose can therefore be the result of repetitive non-allergic stimuli [10, 38].

The role of subepithelial vascularity in the pathophysiology of asthma is also still controversial. Methodological differences have lead to conflicting results. Uneven distribution of vessels, methods of fixation, the influence of tissue oedema and the endothelial marker may affect the final result. Several reports have been published stating that vascularity in asthmatic airways is increased compared to normal controls and correlated with the severity of asthma [12, 39, 40]. However, we could not find any relationship between mucosal vascularity and clinical expression. Our results seem to be more in agreement with those of Carroll, who could also detect no difference in the total number of vessels and vascular area/mm<sup>2</sup> between asthmatic subjects and non-asthmatic controls [11]. We used CD31, an endothelium marker, instead of a collagen marker [12, 39] or factor VIII [11]. In our hands, this marker proved to be superior in the staining of smaller vessels in both upper and lower airways [5].

Eosinophils have long been regarded as the cause of structural changes in the airway mucosa, as they are a source of degranulation products cytotoxic to the epithelial cells. The current study challenges this hypothesis. No association could be found between numbers of inflammatory cells and aspects of airway remodelling. This lack of relationship is particularly clear in nasal mucosa, where we see no difference in epithelial integrity, BM thickness and vascularity, despite increased numbers of eosinophils.

In contrast to local inflammatory and structural factors in nasal and bronchial mucosa, which failed to show a relationship with clinical manifestations, we found a significant correlation

between blood eosinophils and bronchial obstruction (FEV1/VC ratio) and nasal symptom score in atopic patients. In addition, skin test reactivity (atopy score) was significantly related to PC20 methacholine, indicating that allergy is a systemic disease with increased clinical expression (i.e. lower airway involvement) in 'severe' atopic subjects. Our findings are in line with those of Gergen et al. [41] who showed that the prevalence of asthma and rhinitis is positively correlated with the number of positive skin prick test reactions to inhalant allergens. Moreover, Kerkhof et al. found that the presence of BHR or a high blood eosinophil count increased the risk of asthma in atopic subjects [42].

The fact that IL-5 and eotaxin concentrations in serum of allergic patients were not increased compared to healthy controls are not in conflict with our hypothesis. Increased serum-IL-5 and-eotaxin levels have been demonstrated after allergen challenge and during exacerbation of disease [14, 43]. Peak concentrations of serum-IL-5 and-eotaxin are usually reached in 4–12 h after the acute event. Apparently, a pulsatile pattern of chemotactic factors in the serum is sufficient to result in a continuous release of eosinophils from the bone marrow into the bloodstream.

We propose that acute inflammation is related to nasal and/or pulmonary symptoms and influences airway calibre through the release of a variety of mediators. The relationship between chronic inflammation and clinical disease is, however, less straightforward. Generalized airway eosinophilia is a feature of both allergic rhinitis and asthma and not related to clinical severity in stable disease. In both asthmatic and non-asthmatic atopic subjects, structural changes are seen in the lower airways, but not in the upper airways. We could not demonstrate a relationship between aspects of airway remodelling and clinical disease. Our results suggest that systemic factors, such as atopy and blood eosinophils, could be relevant to the clinical expression in allergic airway disease.

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