

Original article

Indices of lower airway inflammation in children monosensitized to house dust mite after nasal allergen challenge

Background: There are few available data assessing the united airway disease and its systemic aspects in children. With this study, we aimed to investigate the inflammation markers of upper and lower airways before and after nasal allergen challenge in mite sensitive children with different clinical expression of the allergic disease.

Methods: Four study groups were formed: rhinitis only, without bronchial hyper-responsiveness (R, $n = 10$), rhinitis with asthma (R + A, $n = 22$), atopic asymptomatics (AA, $n = 8$) and nonallergic healthy controls (C, $n = 10$). Blood eosinophils, nasal and sputum eosinophils, sputum eosinophil cationic protein (ECP) and cys-LTs, and serum ECP levels were measured before and 24 h after nasal allergen challenge.

Results: The groups were comparable in terms of age and gender. Cumulative symptom scores recorded during and 1 h after nasal challenge were not significantly different between patients with R, R + A and AA groups. At T₂₄, the children belonging to R, R + A and AA showed significant increases in nasal eosinophils ($P < 0.01$, $P < 0.001$, and $P = 0.01$, respectively), sputum eosinophils ($P = 0.01$, $P < 0.001$, and $P < 0.05$, respectively) and blood eosinophils ($P < 0.01$, $P < 0.001$, and $P < 0.05$, respectively). Similarly, increases in sputum ECP ($P < 0.01$, $P < 0.001$, and $P = 0.07$, respectively) and sputum cys-LT levels ($P = 0.07$, $P < 0.001$, and $P < 0.05$, respectively) were detected in children belonging to these three groups at T₂₄. Sputum eosinophils significantly correlated with blood eosinophils ($r = 0.54$, $P < 0.001$) and sputum ECP ($r = 0.58$, $P < 0.001$) at T₂₄.

Conclusions: This study showed that nasal allergen challenge increased markers of eosinophilic inflammation in both upper and lower airways of children monosensitized to mites, even before the onset of clinical symptoms.

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Allergic rhinitis and asthma are two entities of the atopic syndrome that often coexist in the same patient (1). The discrimination between allergic rhinitis and asthma can be difficult, especially in childhood, because symptom perception is widely variable; pulmonary function tests can be normal in mild asthmatic children, whereas bronchial hyper-responsiveness (BHR) is frequently present in children with rhinitis only (2).

Studies of induced sputum have indicated that rhinitis with BHR represents an intermediate stage between controls and those with rhinitis and clinical asthma, recorded as induced sputum eosinophils or eosinophil cationic protein (ECP) (3–5).

It has been shown that, although there are no apparent clinical manifestations of lower airway inflammation, inflammatory cells and mediators have been found in the lower airways of allergic rhinitis patients without asthma (3, 6).

Cysteinyl-leukotrienes (cys-LTs) are actively involved in the inflammation seen both in asthma and rhinitis. Cys-LTs are known to be elevated in sputum from asthmatics (7, 8) and have additionally been shown to be correlated with eosinophil count (9).

We designed this study to investigate the difference in lower airway inflammation after nasal allergen challenge in atopic children with various clinical expressions of the allergic airway disease using blood and induced sputum samples.

The aim of this study was to measure induced sputum cys-LTs, as well as ECP as a marker of eosinophilic inflammation before and after nasal allergen challenge in sera and sputum from three groups of allergic children monosensitized to house dust mite: those with perennial allergic rhinitis, with no asthma; those with perennial allergic rhinitis and asthma; and those with just atopy, without any respiratory symptoms. A group of nonallergic healthy children served as controls.

Materials and methods

Participants

The patients for this study were selected among those who were referred to the Pediatric Allergy and Immunology Clinic on the basis of perennial nasal and/or bronchial symptoms. Four study groups were formed: allergic rhinitis, with no asthma (R, $n = 13$); allergic rhinitis and asthma (R + A, $n = 25$); atopic children, with no asthma and no rhinitis (AA, $n = 8$); and nonallergic healthy control children (C, $n = 12$). The participants were interrogated about detailed medical history and followed up at least 1 year prior to the study to obtain optimal inclusion criteria related to their actual diagnosis and to catch an asymptomatic period. All children in the first three groups were sensitized to only house dust mites. Allergy was assessed by skin prick tests. The children were tested with a panel of common inhalant allergens (Dermatophagoides pteronyssinus and farinae, grass mix, tree mix, mold mix, *Alternaria* species, *Cladosporium* species, eucalyptus, olive and dander) (Allergopharma, Reinbeck, Germany). A mean wheal diameter > 3 mm was considered positive. Methacholine challenges of patients with R and AA were negative. Controls had a negative skin prick test, and were not hyper-responsive to methacholine. Asthmatic patients fulfilled the criteria for mild persistent asthma according to GINA guidelines (10). Diagnosis of persistent allergic rhinitis was based on criteria in ARIA (11) consensus statement. The atopy score was determined in this way: 1+, 2+, 3+, or 4+ if the wheal diameter with *D. pteronyssinus* was equal to 25%, 50%, 100%, or 200% of the size of the histamine wheal.

No patient had had an upper respiratory tract infection in the 6 weeks preceding the trial nor used inhaled corticosteroids or leukotriene antagonists or other treatment, apart from short-acting beta-2 agonists for at least 4 weeks before entering the trial. None of the study participants had used oral or systemic corticosteroids 6 months prior to the study.

The study was approved by the ethical committee of the University Hospital and each patient and their caregivers gave written informed consent.

Study design

At the first screening visit, a full history, physical examination, skin prick tests, anterior rhinomanometry, and pulmonary function tests were performed. On the following day, methacholine challenge was performed. One month later, the patients attended a baseline sputum induction; nasal brushing and serum of the patients were collected. The following day, the patients attended for a nasal allergen challenge and 24 h later sputum induction, collection of the sera and nasal brushing samples were repeated.

Methacholine challenge

The challenge was carried out according to ATS guidelines (12) using a five-breath dosimeter (Koko Dosimeter, Ferraris Respiratory, Louisville, KY, USA) with purified methacholine between 2 and 4 PM to avoid circadian rhythm effect on airway dynamics.

Nasal brushing technique

Nasal cytologic specimens were obtained by using an cytologic brush (13). It was introduced in the middle meatus of the nose and turned for 360°; then, it was immediately placed in phosphate-buffered saline (PBS) and incubated for 30 min after shaken

vigorously. Differential cell count was performed on cytopins (Cytospin 4; Shandon Corp., Pittsburgh, PA, USA) stained using May-Grunwald-Giemsa after counting 200 inflammatory cells after centrifugation at 400 g for 10 min. Samples were examined in a blinded fashion.

Nasal provocation

Nasal provocation (NP) was performed according to the EAACI guidelines (14) using a Rhinospir 165 rhinomanometer (Sibelmed, Barcelona, Spain). The response was evaluated by measuring nasal resistance at 150 Pa with active rhinomanometry and by scoring the clinical symptoms. Total symptom scores represented the sum of the scores for: sneezing (0–2 sneezes: 0 points; 3–5: 1 point; > 5 : 2 points); rhinorrhea (moderate: 1 point; severe: 2 points); tearing, itching (eyes, throat); 1 point; conjunctivitis, cough, urticaria and/or dyspnea: 2 points. Positive clinical nasal challenge was defined as > 3 points (15).

After spraying 0.2 ml of the diluent, increasing concentrations of *D. pteronyssinus* allergen extract (50, 500, 5000 AU/ml, Allergopharma, Reinbeck, Germany) were sprayed into the same nostril every 15 min until symptoms appeared and resistances doubled those induced by diluent. If this was not achieved at the highest concentration, the dose was increased by giving two squirts in each nostril. Records of nasal symptoms of nasal blockage, itching, sneezing, discharge were recorded hourly on a seven-point Licert scale (1 = severe symptoms, 7 = no symptoms), which had been previously described (16).

Sputum induction

Sputum was induced by inhalation of a hypertonic saline (4.5%) for 1, 2, 3, 4, 8 and 16 min via an ultrasonic nebuliser (Hikoneb 906S, Kare Medical, Ankara, Turkey) as previously reported (17). Forced expiratory volume (FEV₁) was measured 1 min after each inhalation period. Subjects were asked to rinse their mouth and blow their nose, and try to cough between each dose of nebulized saline. Induction was continued until adequate sputum sample was obtained and stopped when the final inhalation (16 min) given or if the patient developed either a decrease in FEV₁ $> 20\%$, or shortness of breath.

Sputum processing and analysis

As described in previous reports (17), selected portions of the sputum were chosen by using an inverted microscope, and were treated with four volumes of Dithiothreitol (DTT; Sputalysin; Calbiochem Corp., San Diego, CA, USA) which diluted freshly in a 1 : 10 dilution with distilled water. The DTT-sputum mixture was homogenized in a shaking water bath at 37°C for 20 min. Additional four volumes of PBS were added, followed by filtration through 50 µm filter and final centrifugation (790 g for 5 min). The supernatants were frozen at -80°C for later analysis.

Cytospin preparations (Cytospin 4; Shandon Corp.) were made at 220 g for 6 min and stained with May-Grunwald-Giemsa for an overall differential cell count of 400 nonsquamous cells (18). The results are expressed as percentage of total nonsquamous cells.

Cysteinyl leukotrienes in sputum

Total LTC₄, LTD₄, and LTE₄ were measured in the sputum supernatants by enzyme immunoassay, and in a duplicate manner,

employing a cys-LT monoclonal serum (Oxford Biomedical Research Inc., Oxford, MI, USA), after prior purification on C₁₈ Sep-Pak columns (Waters Associates, Milford, MA, USA). Cys-LT concentrations were corrected for sputum dilution during processing, and are expressed as ng/ml of original sputum sample.

Blood eosinophils

The total number of blood eosinophils was determined with hematoxylin-eosin stain by means of hemocytometer.

Serum and sputum ECP

The concentrations of ECP in the sputum supernatant and serum samples were measured with commercially available enzyme assay (CAP-FEIA, Pharmacia, Uppsala, Sweden) with a lower detection limit of 2 µg/l.

Exposure to house dust mite allergens

Dust samples were taken from the mattresses using a vacuum cleaner equipped with a nozzle (Indoor biotechnologies, Manchester, UK) containing a collector with a filter paper (19). A semi-quantitative test (Acarex[®]; Allergopharma, Reinbeck, Germany) was carried out with dust sample to assess the guanine dosage. Exposure was assessed in this way: 'none', 'mild', 'moderate', and 'heavy' (scored as 0, 1, 2, and 3, respectively).

Statistics

All the analyses were performed using computer software (SPSS version 11.0; SPSS; Chicago, IL, USA). Data are presented as median values and min-max or mean ± SD, as indicated. The Wilcoxon signed-rank test for intra group analysis and Mann-Whitney *U*-test for between group analysis if there were two samples were performed. Bonferroni's correction was applied ($P < 0.10/n$; where n = number of comparisons) when multiple comparisons were made and the level of significance was increased ($P \leq 0.02$). Correlations were evaluated with the Spearman correlation test.

Results

Participants

Although 58 children were initially involved at the beginning of the study, the results of only 50 (22 patients with R + A, 10 patients with R, eight atopic asymptomatic and 10 healthy children) were included in further analysis because of unsuccessful induction of sputum or unqualified sputum samples.

A summary of characteristics of participating subjects is presented in Table 1.

There were no statistically significant differences between study groups in terms of age ($P = 0.5$), gender ($P = 0.6$), levels of exposure to house dust mites ($P = 0.5$), FEV₁ values ($P = 0.7$) and total nasal inspiratory flows ($P = 0.8$) (Table 1). Atopy scores of AA group were found significantly lower than the other groups ($P < 0.01$).

Table 1. The characteristics of the participants in the study groups

	R ($n = 10$)	R + A ($n = 22$)	AA ($n = 8$)	C ($n = 10$)
Age, years*	11.4 ± 3.3 (6–15.8)	9.9 ± 2.9 (6–14.5)	10.5 ± 1.9 (8.4–14)	10.9 ± 2.4 (6.5–13)
Gender (M/F)	8/2	15/7	4/4	5/3
Atopy score†	2.4 ± 0.7	2.9 ± 0.7	2.1 ± 0.3	0
Acarex [®] †	1.6 ± 0.9	1.5 ± 0.9	1.2 ± 0.4	1.1 ± 0.7
FEV ₁ (% of predicted)†	95.1 ± 7.4	95.3 ± 10.1	96.1 ± 7.4	108 ± 10.1
TNIF (l/min)†	303.5 ± 177.1	354.4 ± 181.5	355.3 ± 122.4	400.9 ± 187.3
CSS, during NP†	3.9 ± 2.2	4.1 ± 1.6	3 ± 0.7	0.3 ± 0.9
SS, 1 h after NP†	21.1 ± 4.2	21.1 ± 4	24.3 ± 3.7	28 ± 0

*Data are presented as mean ± SD and (min–max).

†Data are presented as mean ± SD.

TNIF, total nasal inspiratory flows; CSS, cumulative symptom scores; SS, symptom scores.

Nasal challenge responses

There were no significant differences in symptom scores recorded during and 1 h after nasal challenge between patients with R, R + A and AA ($P = 0.3$ and $P = 0.2$ respectively) (Table 1).

No AA patients responded to the highest allergen dosage and all were administered additional highest dose of two squirts in each nostril.

The significant drops (20%, 15%, 12%, 16%, 12%) of FEV₁ were observed in one patient 1 h and in four patient 24 h after challenge, respectively; all had rhinitis with asthma.

Nasal eosinophils

Nasal eosinophils increased significantly in patients belonging to R, R + A and AA groups after NP (Table 2). No differences were seen in nasal eosinophil counts between patients with R and AA at T₂₄ ($P = 0.3$).

Sputum eosinophils

The overall sputum cell counts did not significantly change after NP in all groups ($P = 0.8$). Sputum eosinophils were significantly increased in R, R + A and AA groups, after NP (Table 2).

Blood eosinophils

Statistically significant increases were observed in blood eosinophils of patients with R, R + A and AA at T₂₄ (Table 2). No statistically significant differences existed in blood eosinophils between patients with R and R + A ($P = 0.09$), or AA ($P = 0.4$) at T₂₄.

Sputum cys-LTs

At baseline, sputum cys-LT levels were not found significantly different between groups ($P = 0.4$). Sputum cys-

Table 2. Pre- and postchallenge results of nasal, sputum and blood eosinophils

		R	R + A	AA	C	P**
Nasal eosinophil (%)	T ₀	4.25 (1–41) ^{cc,d}	18.25 (1–45.5) ^{aa,bb}	1.5 (0–7)	0.5 (0–1)	<0.001
	T ₂₄	16.5 (6.5–67) ^{cc}	28.25 (2–73.5) ^{bb}	13 (1.5–40.5) ^{ff}	0.5 (0–1)	<0.001
P*		<0.01	<0.001	0.01	0.3	
Sputum eosinophil (%)	T ₀	1.37 (0.4–43) ^{dd}	6.4 (0.5–45.5) ^{aa,bb,e}	0.5 (0–3.5)	0.25 (0–1.5)	<0.001
	T ₂₄	5.8 (0.8–43) ^{dd}	16.9 (2.8–78.5) ^{a,bb,e}	2.12 (0.2–17) ^f	0.12 (0–2)	<0.001
P*		0.01	<0.001	<0.05	0.8	
Blood eosinophil (%)	T ₀	290 (100–850)	375 (50–1800) ^{bb}	250 (100–790)	250 (150–550)	<0.05
	T ₂₄	500 (200–1250) ^d	800 (200–3100) ^{bb}	850 (250–1600) ^f	250 (100–400)	<0.001
P*		<0.01	<0.001	<0.05	0.6	

Data are expressed as medians (min–max).

*P value between pre and postcomparison within each groups (Wilcoxon test).

**P value differences between groups (Kruskal–Wallis test).

^aP < 0.02, ^{aa} P < 0.001, between R + A and AA (Mann–Whitney U-test).

^bP < 0.02, ^{bb} P < 0.001 between R + A and C.

^cP < 0.02, ^{cc} P < 0.001 between R and AA.

^dP < 0.02, ^{dd} P < 0.001 between R and C.

^eP < 0.02, ^{ee} P < 0.001 between R + A and R.

^fP < 0.02, ^{ff} P < 0.001 between AA and C.

Table 3. Pre- and postchallenge results of blood and sputum eosinophil cationic protein (ECP), sputum cys-LT levels

		R	R + A	AA	C	P**
Sputum cys-LTs (ng/ml)	T ₀	11.7 (4.5–14.4)	9.9 (5.4–18)	11.7 (6.3–14.4)	7.2 (4.5–9.9)	0.4
	T ₂₄	13 (6.3–17.1) ^{dd}	14.4 (9–23.4) ^{bb}	13.9 (9–14.4) ^{ff}	7.6 (3.6–11.7)	<0.01
P*		0.07	<0.001	<0.05	0.3	
Sputum ECP (pg/ml)	T ₀	2 (2–171.6)	39.2 (2–346.8)	28 (2–135.6)	2 (2–56)	0.2
	T ₂₄	59.8 (2–451.2) ^d	165.3 (28.6–1224) ^{a,bb,e}	49 (2–280.8)	2 (2–58)	<0.001
P*		<0.01	<0.001	0.07	0.1	
Serum ECP (pg/ml)	T ₀	15.6 (8.7–72.2)	30 (5.3–88.6) ^{a,b}	11.4 (7–46.8)	15 (5.1–28)	<0.05
	T ₂₄	21.6 (8.2–42.6) ^{c,d}	21.1 (10.4–84.9) ^{aa,bb}	7.6 (2–19.7)	12.3 (4.7–29.5)	<0.001
P*		0.4	0.7	<0.05	0.2	

Data are expressed as medians (min–max).

*P value between pre and postcomparison within each groups (Wilcoxon test).

**P value differences between groups (Kruskal–Wallis test).

^aP < 0.02, ^{aa} P < 0.001, between R + A and AA (Mann–Whitney U-test).

^bP < 0.02, ^{bb} P < 0.001 between R + A and C.

^cP < 0.02, ^{cc} P < 0.001 between R and AA.

^dP < 0.02, ^{dd} P < 0.001 between R and C.

^eP < 0.02, ^{ee} P < 0.001 between R + A and R.

^fP < 0.02, ^{ff} P < 0.001 between AA and C.

LT levels increased significantly in patients belonging to R + A ($P < 0.001$) and AA ($P < 0.05$) groups at T₂₄; a borderline significant increase in sputum cys-LTs was observed in patients with R at T₂₄ ($P = 0.07$) (Table 3).

Sputum ECP

Sputum ECP levels of patients with R and R + A increased significantly after NP (Table 3). A borderline significant difference ($P = 0.07$) was found in sputum ECP levels of patients with AA after NP. There was no difference in sputum ECP levels between patients with R and AA ($P = 0.7$) at T₂₄.

Serum ECP

At baseline, serum ECP levels of patients with R + A were found higher than in other groups ($P < 0.05$). No statistically significant increases were observed in serum ECP in all groups at T₂₄ (Table 3).

The correlation between inflammatory markers of blood and sputum

At T₂₄, there was a significant correlation between sputum eosinophils and blood eosinophils ($r = 0.54$, $P < 0.001$). Sputum eosinophils did also significantly

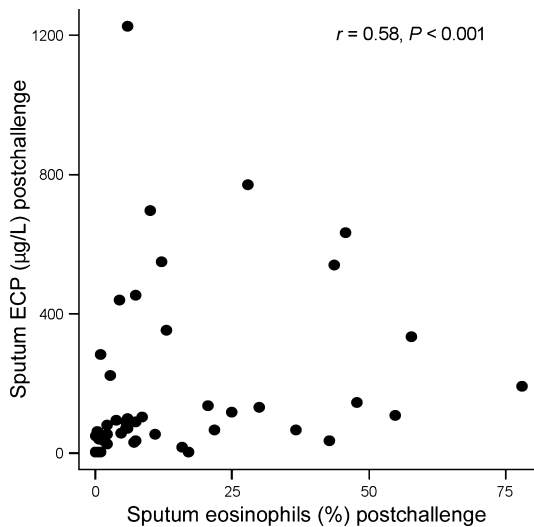


Figure 1. Correlation of sputum eosinophils and sputum eosinophil cationic protein levels after nasal challenge.

correlate with sputum ECP levels at T_{24} ($r = 0.58$, $P < 0.001$) (Fig. 1).

A weak correlation was found between sputum eosinophils and cys-LT levels at T_{24} ($r = 0.26$, $P = 0.07$).

Discussion

In this study, we showed increased cys-LT levels and eosinophilic inflammation in the sputum of allergic children consisting of rhinitis with asthma, nonasthmatic yet with rhinitis, and asymptomatic atopic children, all three groups being monosensitized to house dust mites 24 h following nasal allergen challenge.

There are several investigations analysing cross-talk between the nose and the lungs in allergy via direct pathophysiological mechanism or systemic responses (20, 21). It was reported that treatment of the nose during pollen exposure in patients with rhinitis is also able to improve BHR (22, 23). In a study by Braunstahl et al. (6), there was an increased expression of adhesion molecules and a higher number of eosinophils in bronchial biopsies from rhinitis patients after nasal allergen challenge. Similarly, Beeh et al. (24) demonstrated increases in sputum sICAM and ECP after nasal allergen challenge. Consistent with these studies, we have demonstrated statistically significant or borderline significant increases in blood eosinophils, sputum eosinophils, sputum ECP and cys-LT levels after NP in children with R, R + A and AA groups. The increase in the sputum eosinophils, ECP and cys-LT levels after NP was observed more evidently in children with R + A in comparison with patients with R and AA. These observations supported the studies related to 'united airway disease' or cross-talk between nose and lung.

Actual natural exposure to house dust mites was found to be similar in asthma and the nonasthmatic rhinitis group as well as atopic asymptomatic group. Therefore, the level of exposure to house dust mites does not seem to be the only factor in clinical expression of the allergic disease.

Previously, it was demonstrated that the induction of local allergic inflammation leads to an increased trafficking of eosinophils and progenitor cells from the bone marrow to the peripheral blood and sites of inflammation (25, 26). Our observations, which are a significant increase in blood eosinophils in patients with R, R + A and AA, and a significant correlation between blood eosinophils and sputum eosinophils after NP, support these reports.

Although blood eosinophils and sputum ECP levels of patients with R and R + A significantly increased, serum ECP levels did not increase after NP. It was shown that a majority of eosinophils are transported in the bloodstream in a resting state, and are activated at sites of inflammation (27, 28). Other studies which compared blood eosinophils with induced sputum or BAL, demonstrated increased activation and adhesion markers both in BAL and in sputum (29, 30). Our observation confirms that eosinophils are mainly activated in the tissue of the target organ rather than in peripheral blood.

Although blood eosinophils increased significantly, sputum ECP levels of patients with AA showed a borderline significant difference after NP. This observation may be related to the more exaggerated local inflammatory responses in patients with R and R + A in comparison with patients with AA.

The ability of the potent antagonists at the cys-LT receptor to attenuate both early and late asthmatic reaction to allergen challenge suggests that these mediators play a role in both phases of allergen induced asthmatic reactions. At baseline, we did not find a difference in sputum cys-LTs between patients with rhinitis with or without asthma contrary to what was found by Tufvesson et al. (31), whose study concerned patients with seasonal disease. Our results may reveal that the persistent inflammation may be more of an exaggerated kind in the patients sensitized to house dust mites. In our study, NP produced an increase in the concentration of sputum cys-LTs. Additionally, sputum eosinophils were found correlated with sputum cys-LT levels at T_{24} . This correlation supported the suggestion that eosinophils are a principal source of cys-LTs within the airways.

We found a close similarity in allergic inflammation evaluated in the sputum of atopic asymptomatics and patients with rhinitis only both at baseline and at T_{24} . Braunstahl et al. (32) demonstrated that thickening of the reticular basal membrane and increased epithelial desquamation were present in the lower airways of allergic patients even in the absence of clinical symptoms. Crimi et al. (33) compared the bronchial inflammatory response

following allergen-specific challenge in patients suffering from asthma/rhinitis alone. The authors found no morphological difference between the two groups: the bronchial inflammatory response is the same regardless of which airway is affected by the disease. Our findings which supported such previous reports confirmed that atopic subjects have a common inflammatory response whatever may be the clinical expression of the allergic disease.

It is known that sometimes the discrimination between allergic asthma and rhinitis may be difficult especially in patients with persistent disease. Therefore, a question may arise about categorization of the participants according to their actual diagnosis. Detailed medical history, follow-up at least 1 year prior to the study, clinical and laboratory tests helped with categorization of patients as R, R + A or AA. Although all mild persistent asthmatics had FEV₁ values around 95% of the predicted during the study, it reflects their best values recorded in their asymptomatic period.

We cannot completely exclude the possibility of a contamination of lower airways with nasal secretion through aspiration in children included in this study. Allergen was administered during breath-hold, and patients were carefully advised to keep their heads in a prone position to avoid swallowing or aspiration of

allergen or nasal secretions to the lower respiratory tract during and after challenge. We suggest that aspiration of secretions to the lung might be accepted as negligible in this study as supported by some other studies (34, 35).

There are few reports which investigate the asymptomatic individuals in comparison to patients with rhinitis or asthma. To our knowledge, this is one of the pioneering studies investigating the local or systemic responses to allergen challenge in atopic asymptomatic children. This study revealed significant results about united airway disease as available data assessing the relationship between rhinitis and asthma in childhood are rather scarce. These findings support that allergy is not a disease confined to a specific target organ, but rather a systemic disorder which may have a broad spectrum of clinical manifestations.

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