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Non-eosinophilic asthma in children: relation with airway remodeling

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ABSTRACT

Non-eosinophilic asthma is increasingly recognized as an important clinical-pathological phenotype in adults. However, this entity has been scarcely investigated in children.

In particular, it is unknown whether airway remodeling would develop in children with non-eosinophilic asthma to the same degree as in children with eosinophilic disease.

Toward this aim, we analysed bronchial biopsies from 80 children undergoing bronchoscopy for appropriate clinical indications: 21 with non-eosinophilic asthma, 34 with eosinophilic asthma and 25 control children. Features of airway remodeling (basement membrane thickening, epithelial loss, angiogenesis) and immune activation (inflammatory infiltrate, IL-4, IL-5, TGF-β, TGF-βRII) were quantified by histology and immunohistochemistry.

The main components of airway remodeling were present in children with non-eosinophilic asthma just as in those with eosinophilic disease. Indeed, compared to control children, both non-eosinophilic and eosinophilic asthmatic children had thickened basement membrane, increased epithelial loss and number of vessels. Moreover, in both groups of asthmatics expression of IL-4 and IL-5 was increased, while that of TGF-βRII was reduced, as compared to controls.

This study demonstrates that structural changes typical of asthma develop in asthmatic children even in the absence of a prominent eosinophilic infiltrate, indicating that other mechanisms, besides eosinophilic inflammation, may promote airway remodeling early in life.

Keywords: pediatric asthma, eosinophilia, basement membrane thickening, epithelial damage, angiogenesis.

List of Abbreviations:

BAL: bronchoalveolar lavage

ECP: eosinophil cationic protein

FEV_1: Forced Expiratory Volume in 1 Second

FVC: Forced Vital Capacity

ICS: Inhaled CorticoSteroid

IgE: Immunoglobulin E

IL-4: interleukin 4

IL-5: interleukin 5

TGF-β: transforming growth factor beta

TGF-βRII: transforming growth factor beta receptor type II

INTRODUCTION

Bronchial asthma has become an increasing concern for public health, especially in industrialized countries. The prevalence of asthma has increased significantly over the last 20 years and its incidence has become particularly high in children (1, 2). It is now widely accepted that childhood asthma is more complex than previously recognized, with distinct phenotypes which may differ significantly in terms of aetiology, pathophysiology and clinical outcomes (3-5).

Eosinophils have long been credited with a central role in asthma. Indeed, the pathology of asthma is characterized by a chronic inflammation of the airways, comprising increased numbers of eosinophils, mast cells and T-lymphocytes. This proinflammatory milieu is modulated via Th2 mechanisms (involving IL-4 and IL-5), but recent evidence suggests that other subsets of lymphocytes may be important such as Th17 and Th9, whose activation depends on TGF-β signalling (6). Furthermore the inflammatory reaction is associated with structural changes of the bronchial wall. These changes, collectively termed "airway remodeling", include epithelial loss, basement membrane thickening, smooth muscle increase and angiogenesis.

The relationship between eosinophilic inflammation and airway remodeling is not completely understood; traditionally, remodeling has been considered the unavoidable consequence of long-term airway inflammation. However, the first studies that evaluated the pathology of asthma in children showed that both airway eosinophilia and all the structural changes characteristic of asthma were present in children, even at pre-school age (7-11). These results indicate that the processes leading to remodeling of the airway wall begin early in the course of the disease and most likely occur in parallel with the establishment of chronic inflammation rather than sequential to it.

Moreover, most studies that provided direct evidence for eosinophils as effectors of tissue remodeling were performed in animal models (12,13). Evidence that eosinophils are required for development of airway remodeling is less compelling in humans and, more importantly, it is now well established that human asthma does not necessarily imply eosinophilic inflammation. Indeed, although asthmatics have

eosinophil values in median higher than controls, a significant proportion of patients show no evidence of eosinophilia, despite having all symptoms and functional alterations typical of asthma (14-16).

The issue of non-eosinophilic asthma was first raised in patients with severe disease (17), but there is now more comprehensive information using induced sputum that the non-eosinophilic phenotype is rather common among patients with asthma, not only in those with severe, but also in those with milder forms of the disease (14-16).

As far as we know it has never been investigated whether the eosinophilic and non-eosinophilic patterns of inflammation are associated to different characteristics of airway remodeling in children. Hence, the aim of this study was to investigate the pattern of structural changes (epithelial loss, basement membrane thickening, angiogenesis) and cytokines (IL-4, IL-5, TGF-β and its type II receptor) in children with non-eosinophilic asthma comparing the results to those of children with the eosinophilic form of the disease and control children without asthma.

METHODS

Subjects

Eighty children undergoing fiberoptic bronchoscopy for appropriate clinical indications were recruited in this study: 55 had symptoms of asthma while 25 did not have these symptoms and were included as controls. Children were defined as having asthma when they had repeated episodes of wheezing, breathlessness and cough, particularly at night and early morning, that were present even apart from colds. Moreover, in all asthmatic children, symptoms had to be responsive to bronchodilators. Presence and reversibility of episodic symptoms were assessed by specific questionnaire administered to parents investigating the pattern of symptoms and the clinical benefit of bronchodilators (symptom resolution or significant improvement). This information was confirmed by the child's paediatrician (7,10).

Asthmatic children were then categorized as eosinophilic or non-eosinophilic according to a threshold corresponding to the 90^{th} percentile of the distribution of biopsy eosinophils in controls (28 cells/mm^2, Figure 1). Based on this partitioning, in our population of asthmatic children 34 had eosinophilic asthma (62%) and 21 non-eosinophilic asthma (38%).

Children with eosinophilic asthma underwent bronchoscopy for recurrent pneumonia (n=19), chronic cough (n=10), stridor (n=1), difficult asthma (n=3) or middle lobe syndrome (n=1); children with non-eosinophilic asthma for recurrent pneumonia (n=11), chronic cough (n=7), stridor (n=1), difficult asthma (n=1) or obstructive sleep apnea syndrome (OSAS) (n=1) and control children for recurrent pneumonia (n=12), chronic cough (n=9), stridor (n=3) or laryngomalacia (n=1).

On the basis of the intensity of treatment required to control symptoms, children were considered to have mild, moderate or severe disease (1). Thirty-six out of 55 asthmatic children had mild asthma and were treated with inhaled salbutamol only when needed. Fifteen children had moderate asthma (treated with equivalent daily doses of beclomethasone ranging from 200 to 400 μg) and the remaining 4 had severe asthma (equivalent daily doses of beclomethasone ≥ 800 μg). None of the children were currently being treated with oral corticosteroids or antibiotics at the time of fiberoptic bronchoscopy.

The distribution of mild, moderate and severe asthma was similar in the eosinophilic and non-eosinophilic groups. In particular, mild asthma was present in 20 out of 34 children with eosinophilic asthma (59%) and 12 out of 21 children with non-eosinophilic asthma (57%). Moderate asthma was present in 11 children with eosinophilic asthma (33%) and in 8 with non-eosinophilic asthma (38%). Severe asthma was present in 3 children with eosinophilic asthma (8%) and in 1 child with non-eosinophilic asthma (5 %).

The presence of atopy was defined by an increase in total (paper radioimmunosorbent test [PRIST]) or specific (radioallergosorbent test [RAST]) IgE. In particular, specific IgE for all the following aeroallegens were investigated in all children: house dust mite (Dermatophagoides pteronyssinus, D. farinae), molds (Alternaria alternate), cat dander and grass pollens (Lolium perenne, Poa pratensis, Phleum pratense, Dactylis glomerata, Cynodon dactylon). Control children had to be non-atopic.

The prevalence of atopy was similar in children with eosinophilic and non-eosinophilic asthma; indeed 56% of children with eosinophilic asthma and 52% of those with non-eosinophilic asthma were atopic.

All children underwent routine blood tests, while spirometry was performed only in children who were able to cooperate with the test (n=39). FEV1, FVC and FEV1/FVC were measured using a 10-L bell spirometer (Biomedin, Padova, Italy) and the best of three manoeuvres was expressed as a percentage of predicted reference values.

Bronchoscopy with airway biopsies and bronchoalveolar lavage was conducted according to guidelines (18) and written consent was obtained from children's parents. The study was performed according to the Declaration of Helsinki and was approved by Padova Hospital Ethics Committee (IRB approval n°494P). Some of the children examined in the present study were included in a previous study (10).

Biopsy analysis

Bronchial biopsies were processed as described in the Online-Only Material. Analysis of epithelial loss and reticular basement membrane thickness was performed on sections stained with haematoxylin-eosin. Briefly, epithelial loss was quantified by measuring the length of the incomplete epithelium and

expressed as % of the total basement membrane length. The thickness of the reticular basement membrane was assessed by taking measurements at 50 μm intervals along all the basement membrane length. Vessels were assessed by immunohistochemistry using a monoclonal antibody anti-CD31 as previously described (8), and expressed as number of vessels/mm^2 of examined subepithelium. Analysis of inflammatory cells (eosinophils, neutrophils, mast-cells, macrophages, CD4-T-lymphocytes), as well as of IL-4, IL-5, TGF-β and TGF-βRII was performed by immunohistochemistry as previously described (7,8) and results were expressed as number of positive cells/mm^2 of examined subepithelium. The cases were coded and measurements performed without knowledge of clinical data. Differences were evaluated using the analysis of variance and Student's t-test for clinical data, while the non-parametric Kruskal-Wallis test and Mann-Whitney U test were applied for morphologic data. Correlation coefficients were calculated using Spearman's rank method.

RESULTS

The bronchoscopy procedure was well tolerated by all children and no complications were encountered. The clinical characteristics of the children studied are shown in Table I. Children with eosinophilic asthma were slightly older than children with non-eosinophilic asthma and controls. Importantly, the age at onset of symptoms and symptom duration were not significantly different between the two groups of asthmatics.

FEV1 (% predicted), FVC (% predicted) and FEV1/FVC (%) did not differ significantly between children with eosinophilic and non-eosinophilic asthma. The two groups of children also show a similar degree of reversibility after bronchodilator administration. The levels of circulating eosinophils and BAL ECP were increased in eosinophilic asthmatic children, but not in non-eosinophilic asthmatic children, as compared to control children.

When we examined the different parameters of airway remodeling we found that, not only children with eosinophilic, but also those with non-eosinophilic asthma had a thickened basement membrane

(median, range: 5.4, 2.5-11.5 μm and 5.3, 3.8-8.6 μm vs 3.1, 1.8-4.9 μm; $p<0.0001$ for both), an increased epithelial loss (65, 18-100% and 50, 12-94% vs 33, 0-100%; $p<0.005$ and $p<0.05$ respectively) and an increased number of vessels (259, 9-704 vessels/mm^2 and 250, 0-493 vessels/mm^2 vs 114, 0-576 vessels/mm^2; $p<0.05$ for both) when compared to controls (Figure 2). Examples of these morphological changes in a child with non-eosinophilic and a child with eosinophilic asthma are illustrated in Figure 3.

Furthermore, both eosinophilic and non-eosinophilic asthmatic children had increased numbers of IL-4$^+$ cells (156, 0-676 cells/mm^2 and 117, 0-824 cells/mm^2 vs 56, 8-732 cells/mm^2; $p<0.0001$ and $p=0.007$), as well as IL-5$^+$ cells as compared to controls (310, 0-834 cells/mm^2 and 356, 0-920 cells/mm^2 vs 235, 0-659 cells/mm^2; $p<0.05$ and $p=0.007$) (Figure 4). No significant difference was observed among the three groups of subjects examined in the expression of TGF-β, but both eosinophilic and non-eosinophilic asthmatics had a decreased expression of TGF-β type 2 receptor as compared to controls (48, 0-829 cells/mm^2 and 19, 0-451 cells/mm^2 vs 160, 0-1048 cells/mm^2; $p<0.05$ for both) (Figure 5). No significant differences were observed among the three groups of children as for CD4$^+$T-lymphocytes, neutrophils and mast-cells, while there was a trend for macrophages to be increased, particularly in children with eosinophilic asthma (Table II).

Since the threshold chosen to identify children with eosinophilic and non-eosinophilic asthma was somewhat arbitrary, to validate our results we decided to compare the two extreme subsets: i.e. asthmatic children within the highest quartile of eosinophils (>140 cells/mm^2) and those within the lowest quartile (<21 cells/mm^2). No significant differences were observed between asthmatic children in the highest quartile and those in the lowest quartile as for basement membrane thickness (median, range: 5.9, 3.5-11.5 μm vs 5.4, 3.8-8.6 μm), epithelial loss (63, 18-100% vs 50, 20-94%), vessels (300, 9-620 vessels/mm^2 vs 250, 0-493 vessels/mm^2), IL-4$^+$ cells (208, 0-676 cells/mm^2 vs 101, 56-451 cells/mm^2), IL-5$^+$ cells (303, 0-810 cells/mm^2 vs 323, 0-920 cells/mm^2) and TGF-βRII (47, 0-829 cells/mm^2 and 19, 0-451 cells/mm^2). Importantly, when compared to control children, all the pathological features were present not only in asthmatic subjects in the highest quartile of eosinophils,

but also in those in the lowest quartile (except for IL-5^+ cells, which did not reach levels of statistical significance).

Interestingly, in our study, levels of circulating eosinophils parallel those in bronchial biopsies in the two groups of subjects. In particular, it is worthwhile to note that, among 21 patients considered non-eosinophilic based on tissue analysis, none had peripheral eosinophilia (eosinophil counts in peripheral blood > 450/mm^3). Conversely, among 34 patients considered to be eosinophilic based on tissue analysis, 14 also had peripheral eosinophilia, while 20 did not. These results indicate that when blood eosinophilia is present, then tissue eosinophilia is to be expected, but low levels of blood eosinophils do not exclude airway eosinophilia. Finally, when we compared subjects with concordant tissue/blood eosinophilia vs discordant tissue/blood eosinophilia, there were no pathological or clinical features able to differentiate the two subsets.

Since asthmatic children included in our study had a broad age range (2-15 years), to validate our findings in more homogeneous subgroups, we performed an age-stratified analysis, considering separately children <6 years (n=49) or ≥ 6 years (n=31). First of all, there was no difference in any of the examined parameters (either structural or inflammatory) between asthmatic children at preschool and school-age (Online-Only Material). Moreover, in both age groups, structural and inflammatory parameters were increased in asthmatic children as compared to controls, with no differences between the eosinophilic and non-eosinophilic forms of the disease (Table III, Table IV). When compared to controls, all trends were confirmed numerically but some of the differences did not reach the levels of statistical significance, probably because of the low number of subjects in each subgroup.

Furthermore, to exclude the potential confounding effect of steroid therapy in some patients, we limited our analysis only to asthmatic children who were not being treated with inhaled corticosteroids (n=36). The main results of our study were confirmed (Table E1). Indeed, when we compared among untreated children, those with eosinophilic and those with non-eosinophilic asthma, there were no differences in epithelial loss, basement membrane thickening or angiogenesis nor in cytokine levels.

Since recurrent pneumonia was a frequent indication for bronchoscopy in our study we performed a subanalysis considering only patients without recurrent pneumonia, and all the main messages were confirmed. Indeed, when we compared children with eosinophilic and non-eosinophilic asthma in this population, there were no differences in epithelial loss, basement membrane thickening or angiogenesis nor in cytokine levels (Table E2).

Finally, we evaluated possible relationships between morphological and functional parameters characteristic of the disease. When all asthmatics were considered together, the number of eosinophils was not related to parameters of airway remodeling, but it correlated marginally with the number of macrophages ($p=0.04$, $r=0.28$), with that of circulating eosinophils ($p=0.01$, $r=0.28$), with the expression of IL-4 ($p=0.004$, $r=0.29$) and with the values of FEV1/FVC (%) ($p=0.03$, $r=-0.39$). Neither epithelial loss, nor basement membrane thickening or the number of vessels was related to the values of FEV1/FVC, FVC, FEV1 or bronchodilator reversibility. This lack of correlation was a consistent finding either when we considered all asthmatic children as one group or in the eosinophilic and non-eosinophilic groups considered separately. Other weak correlations were observed, whose correlation coefficients never exceeded 0.5, and are reported for completeness in the Online-Only Material.

DISCUSSION

This study investigated for the first time airway remodeling in children with non-eosinophilic asthma, in comparison with the eosinophilic form of the disease, to elucidate the relationship between airway inflammatory and structural changes in the first years of life. Of interest, it demonstrates that structural changes characteristic of asthma (basement membrane thickening, epithelial loss and angiogenesis) are present not only in children with eosinophilic, but also in those with non-eosinophilic asthma. These results, do not question the importance of eosinophils as effector cells in asthma, but they rather suggest that other pathways may be involved, thus highlighting the complexity of the disease.

Eosinophils have long been credited with a central role in asthma. Indeed, eosinophilia is a known risk factor for the development of respiratory symptoms, and, among asthmatics, it is associated with higher mortality risk, exacerbations, and lung function impairment (19-22). In particular, sputum eosinophilia is associated with lung function decline in asthmatic patients with persistent airflow limitation (23,24) and, more importantly, eosinophilia is able to predict the subsequent development of persistent airway obstruction among adults with an early onset of symptoms (22). On this line, increased sputum and circulating eosinophils are associated with functional impairment even in children with asthma (25). Based on these observations, it could be hypothesised that the relationship between eosinophils and impaired lung function could be mediated by an effect of these inflammatory cells on airway remodeling. Indeed, eosinophils have the potential to cause damage to the epithelium through the release of basic proteins, lipid mediators and reactive oxygen species. Moreover, they could contribute to remodeling of the airway wall through the release of mediators with fibrogenic activity (26,27). However, in our study, structural changes were present in children with non-eosinophilic asthma just as in those with eosinophilic disease, and eosinophilic inflammation was not related to any of the components of airway remodeling. These results suggest that the factors promoting airway remodeling are probably different from those controlling airway eosinophilia. Of interest, not only structural changes, but even the expression of IL-4 and IL-5 were not significantly different between children with eosinophilic and non-eosinophilic asthma, indicating that persistence of eosinophils in the tissue is a

complex process that goes beyond the up-regulation of these Th2 cytokines. It may appear surprising that the expression of IL-4, and particularly IL-5, was up-regulated in children with non-eosinophilic asthma; however, this is consistent with the idea that some patients never exhibit eosinophilia (28), even in the presence of a pro-eosinophilic milieu.

Indeed, the degree of variability in tissue eosinophil counts in our study was considerable: about 60% of asthmatic children had eosinophils above the 90th percentile of controls, while 40% had values below this cut-off. These frequencies are in line with those reported in adults with asthma by studies using induced sputum (14-16), suggesting that the partition we used was indeed appropriate. We should acknowledge that the stability of non-eosinophilic phenotype could be questionable. A recent longitudinal study reported that this pattern was fairly stable (29), while others showed a higher degree of variability (30, 31). Many potential factors could influence variability of cellular counts, with ICS use and active smoking being the most prevalent (16). Of note, the influence of smoking can be considered trivial in our study (no active, very low prevalence of second-hand smoking) and a minority of children was being treated with inhaled corticosteroids (only 4 out of 55 with high doses). Moreover, our results were confirmed even when we excluded treated children from the analysis and when we compared the two extreme subsets for eosinophil distribution, i.e. asthmatic children within the highest quartile to those in the lowest quartile. In fact, the degree of airway remodeling was really the same in asthmatic children with scanty eosinophils and in those in whom eosinophilic infiltration was massive. Overall, as suggested by other observations (32, 33), airway inflammation in asthma seems to be dissociated from functional and structural abnormalities just as in our study inflammation is dissociated from airway remodeling.

Our observations could be relevant in the context of clinical studies testing whether anti-inflammatory therapies would be able to modify the impairment of lung function. For instance, selective removal of eosinophils with a monoclonal antibody against IL-5, though reducing the rate of exacerbations and improving asthma control in patients with refractory eosinophilic asthma, had little effect on functional parameters (34,35). Similarly, steroid therapy does not affect lung function impairment in very young

children (36,37), even if it seems to have a slight effect in adults with recent onset asthma (38), suggesting that childhood asthma is probably different from adult asthma. On the same line, while we found that a thickened basement membrane was present even in children with non-eosinophilic asthma, some studies in adults reported thickening only in patients with prominent eosinophilia (39,40). These observations again support the concept that phenotypes in childhood asthma represent a different population than in adults. Indeed, the lack of eosinophils in adults with asthma has been associated with increased pulmonary neutrophilia (14-17) while, in our study, there was no evidence of neutrophilia in children with non-eosinophilic asthma.

We are well aware that there are diagnostic issues in children, particularly in the youngest ones, because of the multifactorial nature of wheezing (41). We were very careful when assessing the pattern of symptoms that were not purely virus-induced, but rather multitrigger, and had to be responsive to bronchodilators. As recently pointed out (41), there is insufficient evidence in the literature on the pathophysiological mechanisms at preschool age. It is therefore important to highlight that in our study the airway pathology characteristic of asthma was present in children <6 years of age just as in the oldest ones, and that airway remodeling occurred both in children with eosinophilic or non-eosinophilic disease even at preschool age. Of interest, when we examined TGF-β signalling, which plays important regulatory roles in foetal and postnatal lung development, we observed a reduced expression of the type 2 receptor in both eosinophilic and non-eosinophilic asthma. This observation confirms our previous findings in a different subset of children (7), but its significance remains to be clarified. Since TGF-β signalling may affect growth of both epithelium and smooth muscle, it is well conceivable that alterations in this pathway may interfere with the normal maturation of the lung structure.

There are potential criticisms to our study. We acknowledge that the majority of children underwent bronchoscopy for clinical indications other than asthma, otherwise biopsy sampling in children would not be feasible for ethical reasons, and the presence of concomitant diseases could have influenced the results. However, since these conditions were equally distributed among the three groups of subjects, we are confident that they did not affect the observed differences. Moreover, we should admit that a

crucial component of airway remodeling, i.e. the increase in smooth muscle mass, has not been examined in our report. However, because bronchial biopsies sample only a small portion of the bronchial wall, analysis of smooth muscle is not always possible and this is particularly true in children because biopsies are quite small. Lack of more refined functional parameters, such as Rosc or sGAW, which could detect subtle changes in upper airway resistance, is also a significant weakness of the present study (42). Finally, children with non-eosinophilic asthma were not completely devoid of eosinophils, having values marginally higher than controls. However it seems unlikely that the remodeling observed in these subjects was caused by these scanty eosinophils, since all components of airway remodeling were present even in children within the lowest quartile (whose eosinophil values were indeed similar to controls). We should admit that our study gives only a static picture of the inflammation present in airway tissue at a specific time point, and therefore we cannot conclude on the stability of the phenotypes, in particular in relation to exacerbation or remission of symptoms. Non-invasive markers can be evaluated longitudinally; however, analysis of airway biopsies, with all its limitations, gives us the unique opportunity to evaluate the inflammatory pattern exactly in the airway tissue, where structural changes do occur.

In conclusion, this study demonstrates that, even among children, a considerable proportion of asthmatics do not have evidence of tissue eosinophilia. Of interest, the typical airway remodeling is present in children with non-eosinophilic asthma just as in those with the eosinophilic form of the disease. These results suggest that structural changes develop early in the airways of children with asthma, and eosinophilic inflammation is not a necessary requirement.

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FIGURE LEGENDS

Figure 1.

Individual values for eosinophils in bronchial biopsies in asthmatic and control children. Horizontal bars represent median values. The dotted line represents the 90th percentile of eosinophils values in controls (28 cells/mm^2) that was used to subgroup asthmatics in non-eosinophilic and eosinophilic.

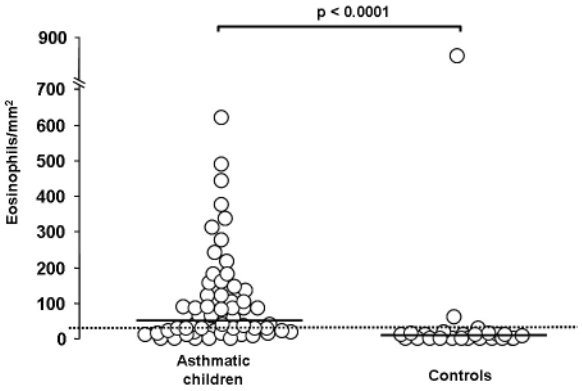
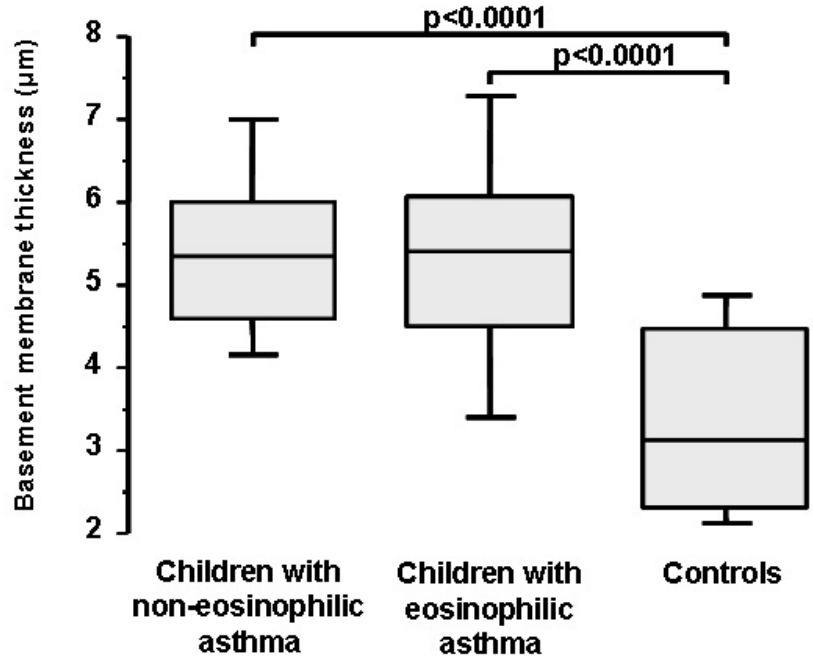
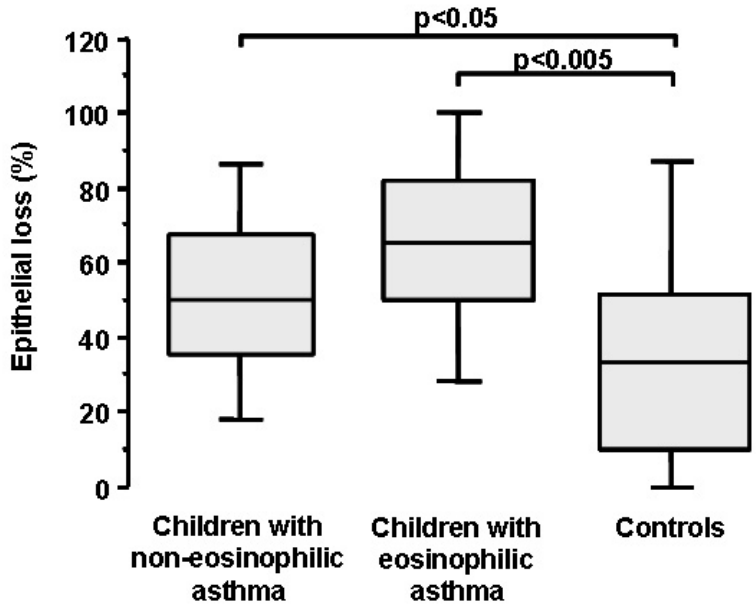


Figure 2.

Individual values for basement membrane thickness (panel A), epithelial loss (panel B) and vessels (panel C) in children with non-eosinophilic asthma, children with eosinophilic asthma and control children. In each box plot, the bottom and the top of the box denote the 25th and 75th percentiles, respectively, the solid line is the median and the brackets are the 10th and 90th percentiles. p values in figure are those of the Mann-Whitney-U-test; Kruskal-Wallis test: $p<0.0001$ (A); $p=0.001$ (B) and $p<0.05$ (C).


p<0.0001
p<0.0001
Basement membrane thickness (µm)
8
7
6
5
4
3
2
Children with non-eosinophilic asthma
Children with eosinophilic asthma
Controls


p<0.05
p<0.005
Epithelial loss (%)
120
100
80
60
40
20
0
Children with non-eosinophilic asthma
Children with eosinophilic asthma
Controls

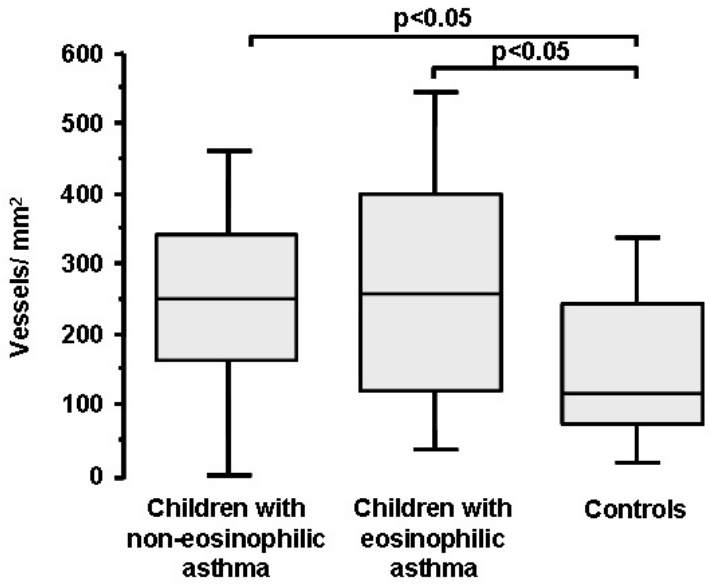


Figure 3.

Bronchial biopsy sections from children with non-eosinophilic asthma (panels A and C) and children with eosinophilic asthma (panels B and D). Arrowheads indicate epithelial loss, whereas arrows indicate reticular basement membrane thickening (panels A and B). Panels A, B: immunostaining with monoclonal antibody anti-EG2 (eosinophils in red). Panels C, D: immunostaining with monoclonal antibody anti-CD31 (vessels in brown). Original magnification x630.

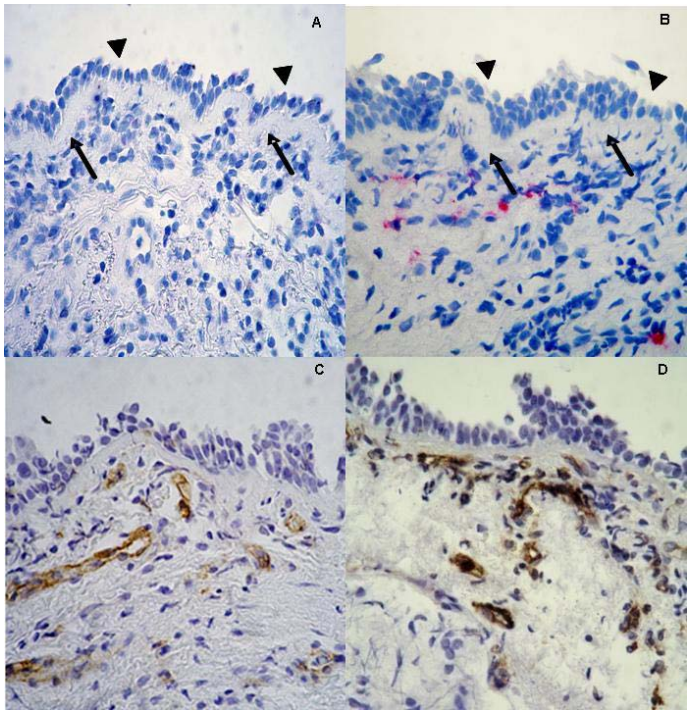


Figure 4.

Individual values for IL-4 positive cells (panel A) and for IL-5 positive cells (panel B) in children with non-eosinophilic asthma, children with eosinophilic asthma and control children. In each box plot, the bottom and the top of the box denote the 25th and 75th percentiles, respectively, the solid line is the median and the brackets are the 10th and 90th percentiles. p values in figure are those of the Mann-Whitney-U-test; Kruskal-Wallis test: p=0.0001 (A) and p=0.002 (B).

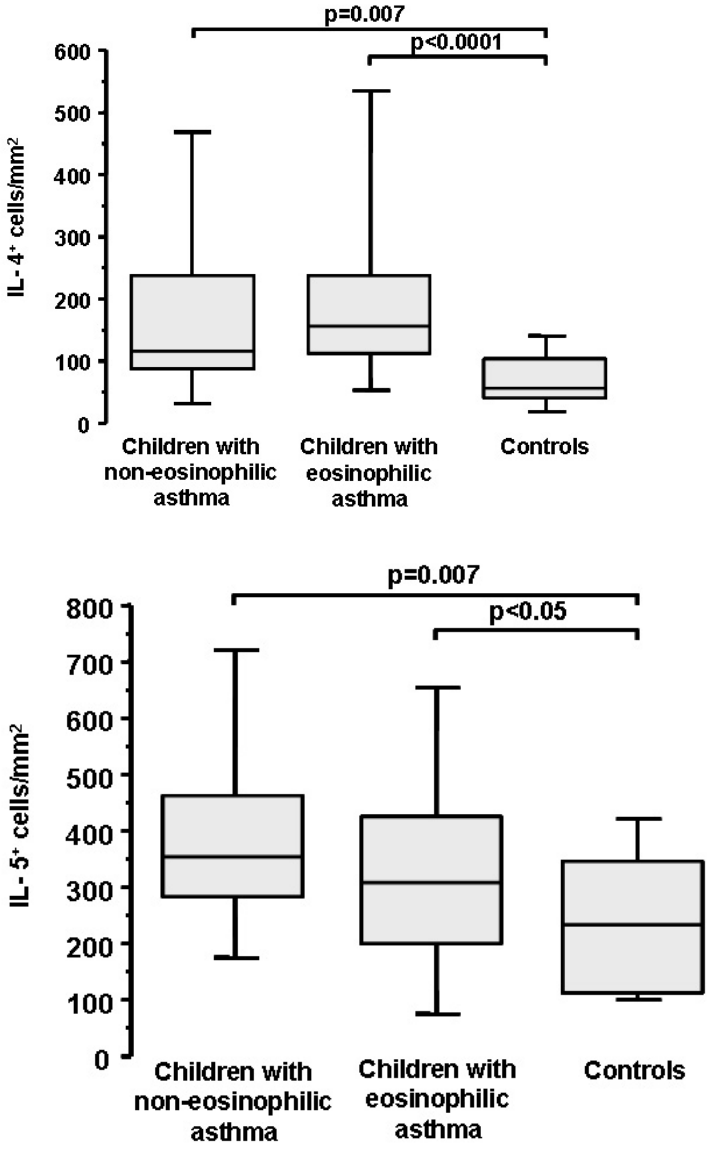
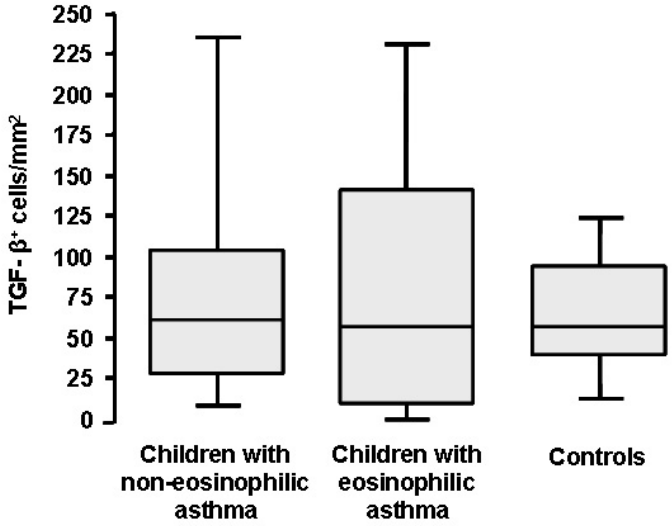


Figure 5.

Individual values for TGF-β positive cells (panel A) and for TGF-β RII positive cells (panel B) in children with non-eosinophilic asthma, children with eosinophilic asthma and control children. In each box plot, the bottom and the top of the box denote the 25th and 75th percentiles, respectively, the solid line is the median and the brackets are the 10th and 90th percentiles. p values in figure are those of the Mann-Whitney-U-test; Kruskal-Wallis test: p=0.05 (B).



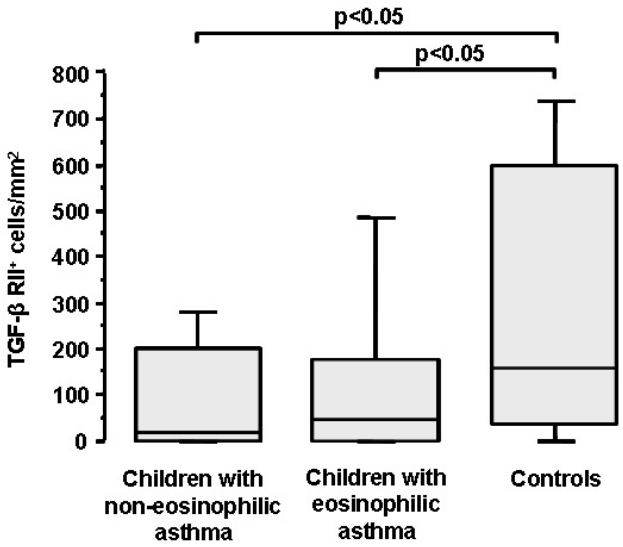


Table I. Clinical characteristics of children

	Children with eosinophilic asthma (34)	**Children with non-eosinophilic asthma (21)**	**Control children (25)**
Number and sex (M/F)	16M/18F	14M/7F	10M/15F
Age *(years)*	7.2±0.5*	5.0±0.5	4.9±0.5
FEV_1 *(% pred)*°	86.4±2.6†	90.7 ± 6.3	101.6±2.5
Δ FEV_1 post-β2*(% baseline)*°	10±2.0 %	11±3.0 %	-
FVC *(%)*°	91.6±2.8	91.7 ± 5.7	100.6±2.5
FEV_1/FVC *(%)*°	85.5±1.8	88.1±2.8	91.7±2.2
Age at onset of symptoms *(years)*	2.8±0.6	2.2±0.4	---
Duration of symptoms *(years)*	4.3±0.6	2.7±0.5	---
Blood eosinophils (*cells/mm^3)*	412±63*	213±25	179±20
BAL ECP *(μg/L)*	71±14†	52±20	16±5
Atopy (yes/no)	19/15	11/10	0/25
ICS treatment (yes/no)	11/23	8/13	---

Data are expressed as mean ± SEM

° Pulmonary function testing was performed in 23 children with eosinophilic asthma, 7 children with non-eosinophilic asthma, and 9 control children. Δ FEV1 post-β2 was available in 20 children with eosinophilic asthma and 5 children with non-eosinophilic asthma.

* $p \leq 0.05$ as compared to children with non-eosinophilic asthma and with control children; † $p<0.01$ as compared to control children;

Table II. Cellular counts in the subepithelium

	Children with eosinophilic asthma	Children with non-eosinophilic asthma	Control children
Eosinophils	111 (29-620)*	13 (0-28)†	7 (0-845)
CD4 T-lymphocytes	308 (0-1546)	202 (0-1423)	244 (0-1826)
Macrophages	113 (0-597)	85 (0-430)	56 (8-732)
Neutrophils	116 (0-1023)	158 (0-611)	134 (0-535)
Mast cells	86 (0-755)	129 (12-575)	56 (6-676)

All values are expressed as median (range); values are expressed as cells/mm^2.

* $p<0.0005$ vs children with non-eosinophilic asthma and vs control children; † $p<0.05$ vs control children.

Table III. Subanalysis in children younger than 6 years.

	Children with eosinophilic asthma (16)	Children with non-eosinophilic asthma (21)	Control children (12)
Epithelial loss, %	55 (20 –100)*	44 (12 – 94)	37 (0 – 100)
Basement membrane thickness, µm	5.1 (2.5 – 7.2)*	5.4 (4.2 – 8.6)*	3.3 (2.1 – 4.9)
Vessels/mm^2	347 (39 – 704)*	252 (0 – 469)*	114 (0 – 574)
IL-4$^+$ cells/mm^2	151 (0 – 676)*	100 (14 – 824)*	56 (14 – 732)
IL-5$^+$ cells/mm^2	322 (0 – 834)	363 (0 – 920)*	183 (0 – 659)
TGF-β$^+$ cells/mm^2	61 (0-354)	75 (0-310)	56 (9-470)
TGF-βRII$^+$ cells/mm^2	75 (0-829)	19 (0-451)	137 (0-810)

Values are expressed as median (range);

* $p< 0.05$ vs controls (Mann-Whitney U test).

Table IV**.** Subanalyis in children ≥ 6 years.

	Children with eosinophilic asthma (18)	Children with non-eosinophilic asthma (7)	Control children (6)
Epithelial loss, %	71 (18 – 100)*	63 (20 – 85)*	6 (0 – 61)
Basement membrane thickness, µm	6.0 (3.7 – 11.5)*	4.6 (3.8 – 6.7)*	2.9 (1.9 – 4.9)
Vessels/mm^2	231 (9 – 620)	245 (0 – 493)	146 (0 – 576)
IL-4$^+$ cells/mm^2	178 (49 – 620)*	145 (0 – 451)	56 (8 – 141)
IL-5$^+$ cells/mm^2	303 (90 – 810)	339 (225 – 416)	268 (89 – 362)
TGF-β$^+$ cells/mm^2	56 (0-282)	47 (7-268)	40 (11-131)
TGF-βRII$^+$ cells/mm^2	46 (0-216)	34 (0-293)	441 (0-1048)

Values are expressed as median (range);

* $p\leq 0.05$ vs controls (Mann-Whitney U test).