AJRCMB Articles in Press. Published on 29-May-2018 as 10.1165/rcmb.2018-0009OC

Clinical and pathologic factors predicting future asthma in wheezing children. A longitudinal study.

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Funding: University of Padova, Italian Ministry of University and Research, Italian Society for Infantile Respiratory Diseases

Running head: transition from early wheezing to asthma

Descriptor number: 1.20 Immunology/inflammation: human studies

Word count text: 3685

AJRCMB Articles in Press. Published on 29-May-2018 as 10.1165/rcmb.2018-0009OC

At a Glance Commentary:

Scientific Knowledge on the Subject: The clinical and pathological factors that could predict the future development of asthma in wheezing children have not been clearly identified.

What This Study Adds to the Field. This prospective study shows that reduced birth weight and multitrigger wheezing in early childhood are clinical predictors of asthma development. It also provides evidence that pathological abnormalities characteristic of asthma, like basement membrane thickening, are present in early childhood and predict future asthma.

"This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org"

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AJRCMB Articles in Press. Published on 29-May-2018 as 10.1165/rcmb.2018-0009OC

ABSTRACT

Rationale: Wheeze is a common symptom in infants, but not all wheezers develop asthma. Indeed, up to 50% of wheezing children outgrow their symptoms by school age. How to predict if early wheeze will become asthma is still a matter of vivid debate.

Aim: To assess the clinical and pathological factors possibly predicting the future development of asthma in children.

Methods: 80 children (mean age 3.8±1 yrs) undergoing a clinically indicated bronchoscopy were followed prospectively for a median of 5 yrs. At baseline clinical characteristics with a particular focus on wheezing and its presentation (episodic, multitrigger) were collected; structural and inflammatory changes were quantified in bronchial biopsies.

Results: Follow-up data were available in 74/80 children. Children presenting with multitrigger wheeze were more likely to be asthmatics at follow-up than those with episodic ($p=0.04$) or without wheeze ($p <0.0001$). Children with asthma also had lower birthweight ($p=0.02$), lower prevalence of breast-feeding ($p=0.02$) and a trend for increased IgE ($p=0.07$) at baseline than those with no asthma. Basement membrane thickness and airway eosinophils at baseline were increased in children who developed asthma at follow-up ($p=0.001$ and $p=0.026$, respectively). Multivariate analysis showed that, among all clinical and pathological factors, multitrigger wheezing, basement membrane thickening and reduced birth weight were predictive of future asthma development.

Conclusions: Multitrigger wheeze and reduced birth-weight are clinical predictors of asthma development. Basement membrane thickening in early childhood is closely associated with asthma development, highlighting the importance of airway remodeling in early life as a risk for future asthma.

Abstract word count: 250

Key words: preschool wheeze, multitrigger/episodic wheezing, asthma outcome, birth weight, basement membrane.

ABBREVIATIONS

AB: antibiotics

BAL: Bronchoalveolar lavage

BD: bronchodilators

BM: basement membrane

FeNO: fractional exhaled nitric oxide

FEV_1: Forced Expiratory Volume in 1st Second

FVC: forced vital capacity

ICS: Inhaled Corticosteroids

IgE: Immunoglobulin E

IL: interleukin

ECP: eosinophilic cationic protein

LRI: lower respiratory infections

OCS: oral corticosteroids

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INTRODUCTION

Asthma is a disease with a highly variable clinical spectrum, in which wheezing is a cardinal symptom (1). Although it is known that asthma is influenced by age, gender, genetic background and environmental exposure, the natural history of the disease is still poorly understood. The current knowledge on evolution from wheeze in early childhood to asthma later on in life originates mainly from epidemiological studies (2-8). However, our understanding of the underlying pathophysiological mechanisms, particularly in the transition from childhood to adolescence, remains incomplete.

Wheezing in infants is a common worrying event for families and pediatricians, because it may herald the development of asthma. Indeed, up to 50% of children experience at least one wheezing episode before the age of three, yet recurrent wheeze in early childhood is not always asthma: about one-half of wheezing preschool children will outgrow their symptoms by school age (3,4,8). The relationship between early wheeze and the future development of asthma is still a matter of vivid debate. A possible way to understand this relationship will be to have a prospective study of wheezing children to determine if wheezing (and, if so, which kind of wheezing, multitrigger or episodic) will predict the future development of asthma. Furthermore, it would be important to know which, if any, airway pathological changes are associated with a particular type of wheezing. While the pathology of asthma is well established, the airway pathology in wheezing children and its potential to predict future development of asthma is less known.

The aim of our study was to investigate the clinical characteristics (with particular focus in the presence and type of wheezing) and the airway pathological features present in early childhood that could herald the development of asthma later on in life. For this purpose we evaluated clinically and pathologically a cohort of 80 children with a mean age of 3.8 years, who had bronchial biopsies while undergoing a clinically indicated bronchoscopy at baseline,

and reassessed them clinically after a median follow-up of 5 years. Some of the results of this study have been reported in abstract form (9,10).

METHODS

Subjects

Children were recruited at Woman's and Child Health Department, University of Padova, Italy, from 2002 to 2014. All children underwent bronchoscopy for appropriate clinical indications according to the ERS guidelines (11), as summarized in Table 1. Fiberoptic bronchoscopy was well tolerated by all children.

Respiratory symptoms (particularly wheezing and its pattern - multitrigger or episodic) were diagnosed at baseline by a respiratory paediatrician. He/she collected a detailed clinical history, visited the child and administered parental interviews focused on the presence of respiratory symptoms, the treatment during the previous 12 months and the presence of allergic manifestations (Table E1, Online Data Supplement).

Episodic and multitrigger wheeze were defined according to the ERS 2008 Task Force: episodic wheeze is wheezing during discrete time periods, often in association with clinical evidence of a viral cold, with absence of wheeze between episodes, while multiple-trigger wheeze is wheezing that shows discrete exacerbations, but also symptoms between episodes (12). Wheezing severity was graded on a scale from 0 to 3 (0: no symptoms; 1: mild; 2: moderate; 3: severe) and its frequency on a scale from 0 to 6 (0: no episodes; 1: <1 episode/month; 2: 1 episode/month; 3: 2-3 episodes/month; 4: 1 episode/week ; 5: >1 episode/week; 6: daily episodes).

At baseline, all children underwent routine blood tests, including complete blood cell count, total and specific IgE (Online Data Supplement). The presence of atopy was defined by an increase in total and specific IgE (IMMunoCAp, Phadia, Sweden). Spirometry was performed only in children who were able to cooperate with the test.

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At follow-up a respiratory paediatrician interviewed the children' parents or the study subjects and conducted a detailed clinical investigation to confirm or exclude the asthma diagnosis. Asthma diagnosis was obtained by the respiratory paediatrician who regularly followed the children during the follow-up and was made according to clinical and lung function criteria as recommended by current guidelines (1,13) in those children with a history of repeated episodes of wheezing, breathlessness or cough, particularly at night or in the early morning, that were present even apart from colds, and were responsive to prescribed bronchodilators. At follow-up visit the typical symptoms (not only wheezing) were to be associated to at least one of the following conditions: a) treatment with regular or as-needed asthma medications and b) presence of airflow obstruction reversible to bronchodilators. At follow-up visit pulmonary function tests (MICRO MEDICAL©, Superspiro, UK) and FeNO measurements (NIOX VERO®, Aerocrine, Solna Sweden) were performed.

Full details of bronchoscopy and bronchial biopsies procedures have been previously described (14,15). Briefly, biopsies were formalin-fixed and paraffin embedded; 5µm thick sections were stained with haematoxylin-eosin to quantify epithelial loss and basement membrane (BM) thickness, and with immunohistochemical techniques to quantify inflammatory cells (eosinophils, neutrophils, mast-cells, $CD4^+$ T-lymphocytes and macrophages, Online Data Supplement). To avoid observer bias, all cases were coded, and measurements made without knowledge of clinical data. Coefficients of variation for repeated measurements reflecting inter and intraobserver variability ranged from 4% to 7% for epithelial loss and BM thickness; from 5 to 10% for inflammatory cells. Written consent was obtained from children's parents. The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova City Hospital.

Statistical analysis

Children characteristics were expressed using mean±SD or median [range] for continuous variables, and counts and percentages for categorical variables. For continuous variables,

normal distributions were tested using the Shapiro-Wilk test. Comparisons among groups were evaluated with either Student's t-tests or Mann-Whitney-U test as appropriate. Distributions of categorical variables were compared with the $\chi 2$-test or with Fisher's exact test, when sample size was small ($n< 5$). Correlation coefficients were calculated using the nonparametric Spearman's rank method. Univariate logistic analyses, followed by a multivariate logistic regression, were performed to detect the strongest predictors of asthma at follow-up. The covariates included in the final models were those that were significantly different between children with and without asthma at follow-up in univariate analyses. All analyses were performed using R (version x64 3.3.3 for Windows) as detailed in the Online Data Supplement. All statistical tests were two-tailed and statistical significance was assumed for P value <0.05.

RESULTS

Clinical characteristics at baseline according to asthma status at follow-up

Our cohort included 80 children all aged ≤5 years (mean 3.8±1 yrs). The median follow-up duration was 5 years (range 1-13 yrs). Figure 1 summarizes the outcome of the study. Follow-up data were available for 74 children: fifty-four out of 80 attended a follow-up visit while 20 children, who lived far from our hospital, completed a questionnaire by telephone and provided available clinical records. The clinical characteristics at baseline of the 6 children lost at follow-up were not different from the remaining 74. At baseline, 38% of children had multitrigger wheezing, 15% had episodic wheezing and 47% had no wheezing.

Table 2 illustrates the clinical characteristics of all children at baseline according to the asthma status at follow-up. At follow-up, 31 children in our cohort had confirmed asthma (42%), while 43 did not (58%). Children with asthma at follow-up did not differ from those without asthma in gender distribution, age at baseline, age at symptoms onset and follow-up duration. Subjects who had asthma at follow-up were more likely to have wheezing at baseline ($p=0.0002$). When

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the pattern of wheezing (multitrigger vs episodic) was examined, children with multitrigger wheezing at baseline were more likely to be asthmatic at follow-up (71%) than children with episodic wheezing (36%) and those without wheezing (20%) (p=0.04; p<0.0001, Fig. 1). Both frequency and severity of wheezing at baseline were higher in children who developed asthma at follow-up (p=0.001; p=0.0008). There was a trend for children who developed asthma at follow-up to have increased IgE levels at baseline (p=0.07) compared to children who did not. Similarly, when we analysed separately children with only 1-2 sensitizations (n=15) and those with multiple sensitizations (n=20), we found that the percentage of children who developed asthma at follow-up was higher in multi-sensitized children (53%) than in mono (bi)- sensitized ones (35%), but the difference was not significant. Of interest, children with asthma at follow-up had a lower birth weight and less breast-feeding than those without asthma (both p=0.02). Pulmonary function parameters and blood eosinophils were not different between the two groups, as was the history of previous bronchiolitis and parental smoking exposure. Seventeen out of 74 children (23%) were treated with inhaled corticosteroids at baseline, with a higher proportion among children who developed asthma at follow-up (42%) than in those who did not (9%, p<0.0001). No difference was observed between the two groups in the proportion of children treated with oral corticosteroids (6% vs 5%) (Table E1, Online Data Supplement).

Figure 2 illustrates the distribution of body weight at birth, showing that children who developed asthma at follow-up had lower birth weight than those who did not, even if the median birth weight values were in the physiological range (panel A). When stratified by the type of wheezing at baseline (panel B), the effect of low birth-weight on the asthma outcome was mostly evident in children with episodic wheezing or no wheezing. Indeed, all children who developed asthma at follow-up in these two groups, were among those subjects with the lowest weight at birth. No such effect was seen in children with multitrigger wheeze (Fig. 2 panel B).

Clinical characteristics at follow-up according to asthma status at follow-up

The clinical characteristics of children with asthma or no asthma at follow-up are reported in Table 3. Age at follow-up was similar in children who developed asthma (8.9±2.9 yrs) and in those who did not (9.5±2.7 yrs). As expected, subjects with asthma at follow-up had increased frequency of wheezing and increased use of as-needed bronchodilators compared to those who did not develop asthma. Overall, asthma was well-controlled in the majority of asthmatic subjects at follow-up: 77% were treated with ICS and used as-needed bronchodilators a median of twice/month. Only one subject had severe asthma and needed treatment with oral steroids. Of note, asthmatic subjects were prescribed more courses of antibiotics and had more lower respiratory tract infections (bronchitis, pneumonia) in the previous year.

In our cohort, most subjects had lung function tests (FEV_1 and FEV_1/FVC) within the normal range and no difference was observed between asthmatic and non–asthmatic subjects at follow-up. Of note, children with asthma at follow-up had significantly increased FeNO values compared to those without asthma (p=0.04). When we performed FeNO analysis in asthmatic children using the previously reported cut off of 20 ppb (16,17) we found that 47% of asthmatic children had FeNO values >20 ppb, while 53% had FeNo <20 ppb. When we compared asthmatic children with FeNo >20 ppb to those with FeNO <20 ppb, no differences were observed in symptoms, FEV_1, FEV_1/FVC and bronchodilator need at follow-up. Similarly, no differences were observed in the pathological measurements at baseline.

Pathology at baseline according to asthma status at follow-up

The results of the quantitative pathology in bronchial biopsies and BAL analysis performed at baseline according to the asthma status at follow-up are shown in Table 4. Of all parameters evaluated only BM thickening (p=0.001) and eosinophils in bronchial tissue (p=0.026) were higher at baseline in children who developed asthma at follow-up compared to those who did not. The percentage of damaged epithelium and the number of neutrophils, macrophages, mast cells and $CD4^+$ lymphocytes in bronchial biopsies were not different in the two groups of

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children, neither were inflammatory cells and mediators in BAL (Table 4). Figure 3 illustrates the distribution of BM thickness at baseline, showing that children who developed asthma at follow-up had thicker basement membrane than those who did not (panels A and B). This finding was confirmed even when only children who were aged 3 years or less were considered (n=24, Table E3 Online Data Supplement). Children with a history of wheezing at baseline, mainly multitrigger wheezing, had significantly thicker basement membrane than non-wheezing children (Fig. 3 panel C). Basement membrane thickness was positively correlated with the frequency ($r=0.49$; $p<0.0001$) and severity of wheezing at baseline ($r=0.4$; $p=0.0005$), and with frequency of wheezing ($r=0.41$; $p=0.0005$) and use of bronchodilators at follow-up ($r=0.26$; $p=0.03$). Since well-preserved airway smooth muscle was present in a minority of subjects in our study (n=10), we could not perform a complete analysis for this parameter. Results of the stereological quantification of smooth muscle volume fraction in this small subset of subjects are reported in the Online Data Supplement.

Figure 4 shows the distribution of tissue eosinophils at baseline, indicating that children who developed asthma at follow-up had higher eosinophil numbers than children with no asthma at follow-up (panel A). When stratified by the pattern of wheezing at baseline, children with multitrigger wheeze, but not children with episodic wheeze, had an increased airway eosinophilia compared to non-wheezing children (panel B). Of interest, children with episodic wheezing at baseline had increased epithelial damage and increased number of mast cells in bronchial biopsies compared to non-wheezing children (Table E4 Online Data Supplement). When we examined the relationship between number of eosinophils in airway tissue and number of eosinophils in blood, we found a weak, even if significant correlation ($r= 0.24$, corresponding to r squared=0.06), indicating that blood eosinophils cannot be considered representative of tissue eosinophils in our population (Fig. 5). No correlations between blood and tissue eosinophils were observed when only values above or below the normal limits (18) were considered.

A subset of children included in our study (n=17; 23%) was treated at baseline with inhaled steroids, which could have influenced the relationship between pathological changes and asthma outcome. When we restricted our analysis to children not treated with inhaled steroids (n=57) the main messages were confirmed (baseline BM thickness and airway eosinophilia were increased in subjects with asthma at follow-up; $p<0.05$ for both).

A logistic regression analysis of our data at baseline was performed to determine which, among all the variables, were related to the development of asthma at follow-up (Table 5). Then a multivariate analysis showed that among the clinical factors multitrigger wheeze and low birth weight, and among the pathological factors BM thickening were the factors at baseline that would predict the eventual development of asthma in our cohort (Table 5).

Discussion

Wheezing in early childhood is a common worrying event for families and paediatricians alike, because it might herald the development of asthma. However, there is the possibility that wheezing could be benign and not a sign of early asthma presentation.

In an attempt to clarify the meaning of childhood wheezing we prospectively studied a cohort of children who had bronchial biopsies, in whom we assessed clinical characteristics (with a particular focus on wheezing), airway pathology and eventual asthma development. Our results showed that multitrigger wheezing, basement membrane thickening and reduced birth weight were significantly associated with the development of asthma at follow-up.

We defined the wheezing pattern in our children cohort using the two symptom-based phenotypes of wheeze: episodic, which is triggered mostly by viral respiratory infections with affected children symptom-free between episodes, and multitrigger wheeze, triggered by viruses and other causes like allergens, characterized by presence of symptoms between episodes (12). Once the wheezing pattern was defined and bronchial biopsies obtained, we followed our cohort for a median of five years after our first evaluation and found that 71% of

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multitrigger wheezers become asthmatics at follow-up while only 36% of the episodic wheezers do. These data, obtained in a prospective evaluation of our cohort, highlight the importance of recognizing this symptom early in childhood and confirm previous evidence that episodic wheeze frequently undergoes remission, while multitrigger wheeze is more likely to be persistent (19). Furthermore, there are differences in airway function (i.e. conductive airways ventilation inhomogeneity) between multitrigger and episodic wheezers (20), which may indicate that they reflect different disease entities.

Although it has been suggested that the episodic/multitrigger classification is not stable over time (21,22), recent evidence from 2 large longitudinal cohorts suggests that multitrigger, and to a lesser extent episodic wheeze, tend to persist longitudinally regardless of wheezing severity (23). Our results based on clinical information, which included both type and severity of wheezing, carefully collected at a single visit, demonstrated that wheezing information can be valuable and predictive of future development of asthma.

Among the other clinical factors examined, we observed that low birth weight and reduced breast-feeding were associated with established asthma at follow-up in our study cohort. Children who developed asthma at follow-up had both a significantly lower birth weight, approximately 500 g less, and significantly less breast-feeding than children with no asthma at follow-up. A number of other studies have shown similar associations, although this had not been consistently replicated (24-28). The findings in our study underline the important contribution of birth weight in the mechanism of asthma development, especially in children with episodic wheeze or no wheezing (Fig. 2). The mechanisms underlying the associations of low birth weight with asthma outcomes are yet incompletely understood. Low birth weight as a consequence of low gestational age might have a long-lasting impact on the structure of the airways and the lung, which could predispose along with other factors to the development of increased airway reactivity and asthma.

Children who developed asthma also tended to have higher IgE levels than those who did not. This confirms the role of atopy as predictor of asthma persistence throughout childhood and adolescence (3,29), directly through early allergen sensitization but also indirectly impairing immune response to viral infections (30,31).

In order to better understand if wheezing and asthma development at follow-up were linked to airway pathology at baseline, we investigated prospectively children who had to have diagnostic bronchoscopies. All children in our cohort, who had a mean age of 3.8 years, had bronchoscopies performed for appropriate clinical indications, according to international guidelines (11). The bronchial biopsies obtained, which were ethically approved and consented, have provided a pathological basis for the clinical and wheezing characteristics and their association with future asthma outcomes.

Basement membrane thickening at baseline was significantly increased in children who developed asthma at follow-up than in children who did not. Importantly, when our cohort was stratified by age, basement membrane thickness was the only factor to be associated with asthma at follow-up in children who were 3 years or younger at baseline. The multivariate analysis of our data showed that basement membrane thickening, along with multitrigger wheezing and low birth weight, was a predictor of asthma development later in life.

Our results differ substantially from previous studies (32-34) in which bronchial biopsies in asthmatic children were also performed, but basement membrane thickening was not found to predict asthma development. Possibly these differences are due to the dissimilar populations studied. The children in those studies were younger, had severe airway obstruction and were all treated with high dose corticosteroids at the time of bronchoscopy (32-34). By contrast, children in our study were older, their asthma was not severe, and mostly were not treated with corticosteroids at the time of bronchoscopy. When our data was reanalysed excluding the few children on corticosteroids, our results did not change.

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The number of eosinophils in bronchial tissue at baseline was also significantly higher in children who developed asthma than in children who did not. When we examined the relationship between tissue and blood eosinophilia in the whole group of children, although the correlation reached the level of significance, its coefficient was very low ($r=0.24, r^2=0.06$) to reliably predict tissue eosinophilia from blood eosinophils. Our findings are in agreement with other studies correlating blood and airway inflammation (35, 36) and indicate that attempts to infer the presence of airway eosinophilia from blood eosinophil numbers to guide therapy should be contemplated with caution.

In our study we could also compare the airway pathology of multitrigger and episodic wheezing. It has been questioned whether multitrigger and episodic wheezing are truly different conditions, with different pathogenetic mechanisms, or whether they are simply different severity classes of the same underling conditions (21,37-39). Our results of a similar pathology substrate in the two forms of wheezing supports the latter conclusions.

A possible limitation of our study could be that the cohort of children who underwent a clinically indicated bronchoscopy may not be representative of wheezing children in general, since the concomitant diseases could have influenced the results. However, these concomitant conditions were evenly distributed among study groups (Table 1), and most likely did not affect the observed differences.

Unfortunately, at variance with previous studies (32) we were unable to provide a complete assessment of smooth muscle mass, a crucial component of airway remodelling in asthma. The lack of smooth muscle in the majority of the biopsies might be due to the limited depth of the bronchial wall sampled, but also to the mild severity of asthma in our cohort. Indeed, studies in adult asthma have shown that in severe asthma, but not in milder disease, airway smooth muscle gets close to the basement membrane, and it could be more easily sampled by endobronchial biopsies (40).

It should be acknowledged that, even if the differences in basement membrane thickness (p=0.001) and tissue eosinophils (p=0.026) between asthmatic and non asthmatic children were statistically significant, there was a considerable overlap at the individual levels between the two groups (Fig. 3B and 4A), as can be expected in any credible biological measurements. Therefore, these measurements should not be used as predictors at the individual levels but can be of pathological importance in the comparison between groups, contributing to further elucidated the early mechanisms of asthma. Finally, a potential limitation of the study is that we did not performed any mechanistic analyses of bronchoalveolar lavage (BAL) since the study was focused mainly on bronchial biopsies and on measurements of airway pathology.

In conclusion, our study shows that multitrigger wheeze in early childhood and reduced birth weight are clinical predictors of asthma development later in life. Thickening of the basement membrane in early childhood is a pathological finding closely associated with the development of asthma, highlighting the importance of airway remodelling in early life as a risk for future asthma development.

ACKNOWLEDGEMENTS

The authors thank Dr Silvia Ferrarese and Roberta Cenedese for their assistance in follow-up visits.

AJRCMB Articles in Press. Published on 29-May-2018 as 10.1165/rcmb.2018-0009OC

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FIGURE LEGENDS

Figure 1

Flow diagram of the children included in our study stratified according to the presence and type of wheezing (multitrigger/episodic/no wheeze) at baseline. The overall attendance rate was 92.5%.

Figure 2

Panel A: Frequency distribution curves for the values of birth weight in children with asthma (orange) and no-asthma (blue) at follow-up. Panel B: Scatterplot reporting birth weight in children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze). The dashed line represents the median birth weight value for asthmatic children; the dotted line the median birth weight value for non-asthmatic children. Data for this analysis were available in 46 out of 74 children.

Figure 3

Panel A: Frequency distribution curves for the values of basement membrane (BM) thickness in children with asthma (orange) and no-asthma (blue) at follow-up. Panel B: Scatterplot reporting basement membrane (BM) thickness in children with asthma (orange) and no-asthma (blue) at follow-up. Panel C: Scatterplot reporting basement membrane (BM) thickness in children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze).

The dashed line represents the median BM value for asthmatic children; the dotted line the median BM value for non-asthmatic children. Data for this analysis were available in all children (n=74).

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Figure 4

Panel A: Scatterplot reporting eosinophils in bronchial biopsies in children with asthma (orange) or no-asthma (blue) at follow-up. Panel B: Scatterplot reporting eosinophils in bronchial biopsies in children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze).

The dashed line represents the median eosinophil value for asthmatic children; the dotted line the median eosinophil value for non-asthmatic children. Data for this analysis were available in 68 out of 74 children.

Figure 5

Relationship between eosinophils in blood and eosinophils in tissue (bronchial biopsies) at baseline in all the children of our cohort. Spearman's rank correlation coefficient p=0.046; r=0.24 (r^2 = 0.06). Red line represent cut-off for normal values of eosinophils in tissue (23 cells/mm^2) and in blood (400 cells/µL). No correlations between blood and tissue eosinophils were observed when only values above (p=0.7; r=0.09) or below (p=0.7; r=0.06) the normal limits were considered.

Table 1. Clinical indications to bronchoscopy (according to the ERS Guidelines, ref. 11)

Indications	**Whole Cohort *** *n=80*	**Asthma at follow-up** *n=31*	**No asthma at follow up** *n=43*
Recurrent/persistent pneumonia	37 (46%)	13 (42%)	20 (47%)
Chronic cough	17 (21%)	6 (19%)	11 (25%)
Severe therapy-resistant wheezing	11 (14%)	8 (25%)	2 (5%)
Stridor	8 (10%)	2 (7%)	5 (12%)
Suspected tracheomalacia	4 (5%)	2 (7%)	2 (5%)
Foreign body inhalation	1 (1%)	0	1 (2%)
Asphyxia episodes	1 (1%)	0	1 (2%)
Interstitial pnuemonia	1 (1%)	0	1 (2%)

*6 out of 80 children were lost during the follow-up.

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Table 2. Clinical characteristics at baseline in relation to asthma at follow-up

	Whole cohort	**Asthma** at follow-up	**No asthma** at follow-up	*P-value*
Subjects, n (%)	74	31 (42%)	43 (58%)	
Gender, male (%)	38 (51%)	16 (51%)	22 (51%)	n.s.
Age (yrs)	3.8 ± 1	3.7 ± 1	3.8 ± 0.9	n.s.
Age symptom onset (yrs)	1.6 ± 1.13	1.5 ± 1.1	1.9 ± 1.1	n.s.
Follow-up duration (yrs)	5.5 ± 2.6	5.2 ± 2.5	5.7 ± 2.8	n.s.
Wheezing at baseline, n (%)	39 (53%)	24 (77%)	15 (34%)	0.0002
Multitrigger **Episodic**	28 (38%) 11 (15%)	20 (83%) 4 (14%)	8 (53%) 7 (47%)	0.04
Wheezing Severity (0-3)	0 [0-3]	1 [0-3]	0 [0-3]	0.0008
Wheezing Frequency (0-6)	0 [0-6]	3 [0-6]	0 [0-5]	0.001
FEV_1 (% pred) ¶	103 ± 12.2	103 ± 11	103 ± 13	n.s.
FEV_1/FVC (%) ¶	93 ± 7	93 ± 6.5	93 ± 8	n.s.
Blood IgE (kU/L)	47 [0 - 3647]	69 [0-3647]	38 [0-2188]	0.07
Blood eosinophils (cell/µL)	236 [0 - 1760]	310 [0-1760]	210 [0-990]	n.s.
Birth weight (g) *	3386 ± 661	3092 ± 631	3542 ± 632	0.02
Bronchiolitis, n (%) #	19 (32%)	7 (32%)	12 (32%)	n.s.
Breast feeding >3mo, n (%)†	32 (60%)	7 (39%)	25 (71%)	0.02
Parental smoking, n (%) ‡	18 (45%)	7 (47%)	11 (44%)	n.s.

Data are expressed as counts (percentages); mean±SD or median [range]. FEV_1: Forced Expiratory Volume in 1st Second; FVC: forced vital capacity.

p- values refer to the comparison between children with asthma and those with no asthma at follow-up.

Data at baseline available for a subset of children: (¶17 out of 74; * 46/74; # 59/74; † 53/74; ‡ 40/74).

Table 3. Clinical characteristics at follow-up in relation to asthma at follow-up

	Whole cohort	**Asthma** at follow-up	**No asthma** at follow-up	*P-value*
Subjects, n (%)	74	31 (42%)	43 (58%)	
Age at follow-up (yrs)	9.32 ± 2.8	8.9 ± 2.9	9.5 ± 2.7	n.s.
Wheezing Frequency (0-6)	0 [0-6]	1 [1-6]	0 [0-0]	<0.0001
Prescribed ICS (%)	30 (41%)	24 (77%)	6 (13%)	<0.0001
As needed BD (n/mo)	0 [0-30]	2.25 [0-30]	0 [0-5]	<0.0001
Last year AB rounds (n)	0.9 ± 1.2	1.4 ± 1.4	0.5 ± 0.79	0.01
Last year LRI	1.2 ± 2.0	1.93 ± 2.6	0.7 ± 1.17	0.001
FEV_1 (% pred.) ¶	93 ± 16	93 ± 19	93 ± 14	n.s.
FEV_1/FVC (%) ¶	86 ± 7	83 ± 8	88 ± 7	n.s.
FeNO (ppm)*	20 ± 14	27 ± 18	16 ± 11	0.04

Data are expressed as counts (percentages); mean±SD or median [range].
ICS: inhaled corticosteroids; BD: bronchodilators; AB: antibiotics; LRI: lower respiratory infections; FEV_1: Forced Expiratory Volume in 1st Second; FVC: forced vital capacity; FeNO: fractional exhaled nitric oxide.
p- values refer to the comparison between children with asthma and those with no asthma at follow-up.
Data available for a subset of children: (¶ 53 out 74 children; * for 49 out 74 children)

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Table 4. Pathological characteristics at baseline in relation to asthma at follow-up.

	Asthma *at follow-up* n = 31	**No asthma** *at follow-up* n = 43	*P-value*
Epithelial loss (%) *n = 74/74*	65 [12 – 100]	45 [0 – 100]	n.s.
BM thickness (µm) *n = 74/74*	4.6 [3.12 – 8.08]	3.9 [2.1 – 7.4]	0.001
Eosinophils (cell/mm²) *n = 68/74*	61 [0 – 455]	14 [0 – 304]	0.026
Neutrophils (cell/mm²) *n = 70/74*	120 [0 – 1023]	197 [0 – 925]	n.s.
Mast-cells (cell/mm²) *n = 63/74*	271 [28 – 950]	144 [0 – 800]	n.s.
Macrophages (cell/mm²) *n = 67/74*	92 [0 - 597]	118 [0 - 467]	n.s.
CD4⁺Lymphocytes (cell/mm²) *n = 67/74*	312 [0 -1014]	200 [0 -1108]	n.s.
BAL eosinophils (%) *n = 69/74*	0 [0 – 10]	0 [0 – 9]	n.s.
BAL neutrophils (%) *n = 69/74*	17 [0 – 68]	14 [0 – 91]	n.s.
BAL lymphocytes (%) *n = 68/74*	5 [1 – 35]	6 [0 – 26]	n.s.
BAL macrophages (%) *n = 68/74*	75 [22 – 97]	74 [4 – 100]	n.s.
ECP (µg/L) *n = 71/74*	14 [2 – 200]	8 [2 – 200]	n.s.
IL-8 (pg/mL) *n = 51/74*	262 [2 – 3000]	306 [36 – 5728]	n.s.

Data are expressed as median [range]. BM: basement membrane; BAL: bronchoalveolar lavage; ECP: eosinophilic cationic protein.

Table 5. Logistic regression analysis in relation to asthma at follow-up

	Univariate logistic regression			Multivariate logic regression analysis		
	ORs	IC 95%	P-Value	ORs	IC 95%	P-Value
Clinical Factors						
Wheezing pattern (multitrigger)	10.1	3.2-32.1	0.0001	6.5	3.4-28.6	0.02
Wheezing Severity (0-3)	2.9	1.5-5.6	0.001	0.8	0.2-4.5	n.s.
Birth weight (< 3000 g)	6.5	1.5-27.4	0.01	10.3	1.5-27.5	0.01
Breast feeding (> 3 months)	0.3	0.1-0.8	0.02	0.5	0.1-2.0	n.s.
Pathological Factors						
BM thickness (µm)	1.8	1.1-2.8	0.01	1.7	1.1-2.9	0.01
Biopsy Eosinophils (cell/mm^2)	2.3	1.1-4.6	0.02	1.6	0.9-3.1	n.s.

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Figure 1

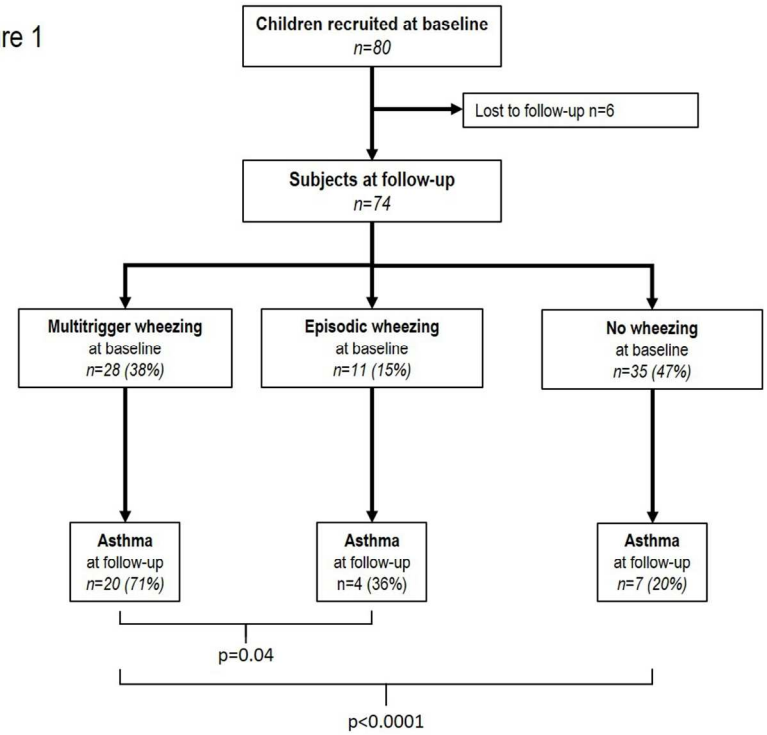

Figure 1. Flow diagram of the children included in our study stratified according to the presence and type of wheezing (multitrigger/episodic/no wheeze) at baseline. The overall attendance rate was 92.5%.

210x180mm (150 x 150 DPI)

Figure 2

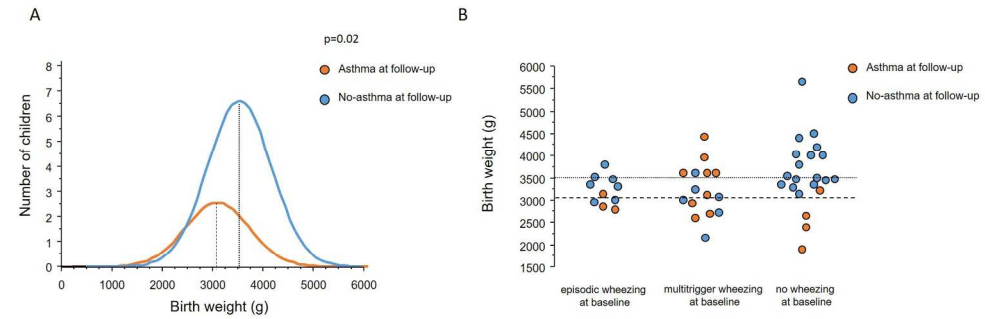

Figure 2. Panel A: Frequency distribution curves for the values of birth weight in children with asthma (orange) and no-asthma (blue) at follow-up. Panel B: Scatterplot reporting birth weight in children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze). The dashed line represents the median birth weight value for asthmatic children; the dotted line the median birth weight value for non-asthmatic children. Data for this analysis were available in 46 out of 74 children.

337x140mm (150 x 150 DPI)

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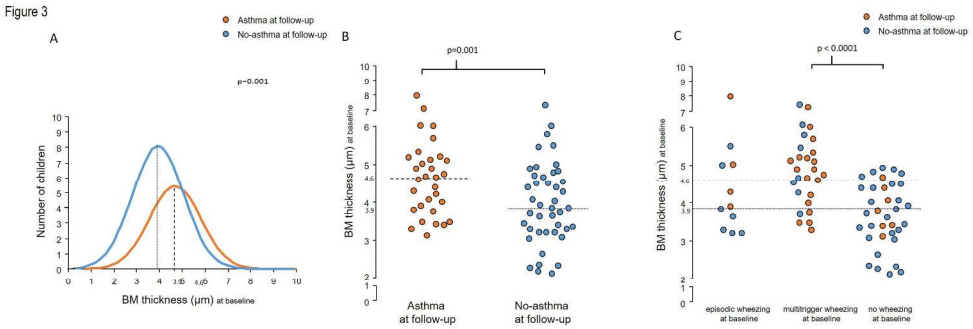

Figure 3. Panel A: Frequency distribution curves for the values of basement membrane (BM) thickness in children with asthma (orange) and no-asthma (blue) at follow-up. Panel B: Scatterplot reporting basement membrane (BM) thickness in children with asthma (orange) and no-asthma (blue) at follow-up. Panel C: Scatterplot reporting basement membrane (BM) thickness in children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze).
The dashed line represents the median BM value for asthmatic children; the dotted line the median BM value for non-asthmatic children. Data for this analysis were available in all children (n=74).

447x176mm (150 x 150 DPI)

Figure 4

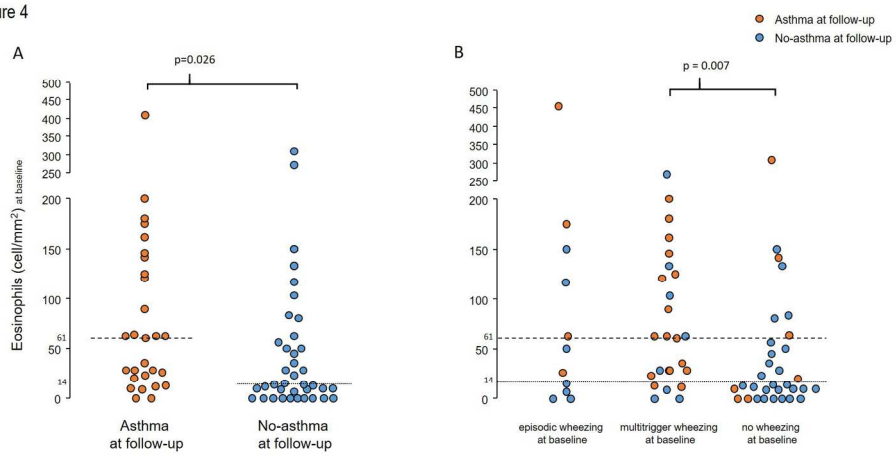

Figure 4. Panel A: Scatterplot reporting eosinophils in bronchial biopsies in children with asthma (orange) or no-asthma (blue) at follow-up. Panel B: Scatterplot reporting eosinophils in bronchial biopsies in children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze).
The dashed line represents the median eosinophil value for asthmatic children; the dotted line the median eosinophil value for non-asthmatic children. Data for this analysis were available in 68 out of 74 children.

341x171mm (150 x 150 DPI)

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Figure 5

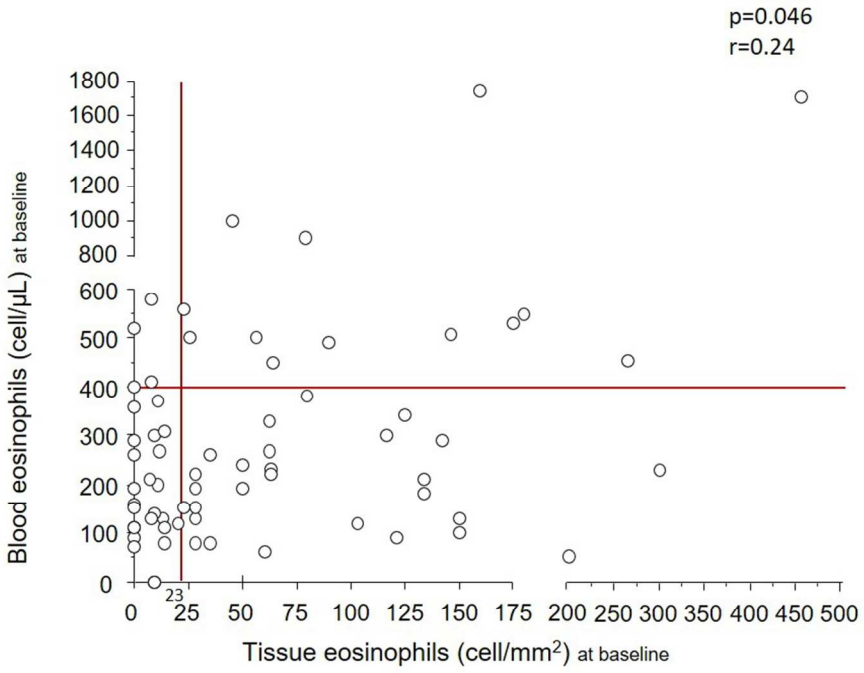

Figure 5. Relationship between eosinophils in blood and eosinophils in tissue (bronchial biopsies) at baseline in all the children of our cohort. Spearman's rank correlation coefficient p=0.046; r=0.24 (r2 = 0.06). Red line represent cut-off for normal values of eosinophils in tissue (23 cells/mm2) and in blood (400 cells/µL). No correlations between blood and tissue eosinophils were observed when only values above (p=0.7; r=0.09) or below (p=0.7; r=0.06) the normal limits were considered.

167x140mm (150 x 150 DPI)

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Online Data Supplement

Clinical and pathologic factors predicting future asthma in wheezing children. A longitudinal study.

Matteo Bonato MD, Erica Bazzan PhD, Deborah Snijders MD, Mariaenrica Tinè MD, Davide Biondini MD, Graziella Turato PhD, Elisabetta Balestro MD, Alberto Papi MD, Manuel G. Cosio MD, Angelo Barbato MD, Simonetta Baraldo PhD, Marina Saetta MD.

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Bronchoscopy and biopsy processing

Full details of bronchoscopy and bronchial biopsies procedures have been previously described (E1-E6). Briefly, bronchoscopy, with endobronchial biopsy and bronchoalveolar lavage, was performed using a flexible bronchoscope with an external diameter of 4.9 mm (E1-E3). One bronchial biopsy specimen was taken using the bronchial forceps Olympus FB 19 C-1 inserted through the service channel of the bronchoscope (2.0 mm diameter). Biopsies were gently extracted, fixed in 4% formaldehyde and dehydrated through alcohol series. They were embedded in paraffin wax and processed for histochemical and immunohistochemical analysis as previously described (E4-E6). Analysis of epithelial loss and reticular basement membrane thickness was performed on sections stained with haematoxylin-eosin. The length of the incomplete epithelium, as well as the total length of epithelium (as assessed by the length of total basement membrane), were measured and epithelial loss was expressed as % of the incomplete epithelium length over total basement membrane length. The epithelium was considered incomplete when the basement membrane was completely denuded or when it was covered by a single layer of basal cells with no intact ciliated cells or goblet cells. The thickness of the reticular basement membrane was assessed by taking measurements at 50 μm intervals along all the basement membrane. Well-defined airway smooth muscle bundles, which area could be stereologically quantified, were present in a minority of subjects in our cohort (n=10). We stereologically quantified by point counting the fraction volume of smooth muscle in these 10 cases, obtaining a median volume smooth muscle fraction Vv of 0.15 (0.03 to 0.3). Of the 10 children examined, only 2 were asthmatic at follow-up and both had Vv values above the median (Vv: 0.28 and 0.19).

Inflammatory cells (eosinophils, neutrophils, mast cells, macrophages, and CD4+ T-lymphocytes) were quantified in the area extending 50 μm beneath the reticular basement membrane and expressed as number of positive cells/mm^2 of subepithelium. Briefly, mouse monoclonal antibodies were used for identification of eosinophils (anti-Eosinophil Cationic

Protein, Diagnostic Developments, Uppsala, Sweden), neutrophils (anti-elastase M752), mast cells (anti-tryptase M7052), macrophages (anti-CD68 M814) and CD4+ cells (anti-CD4 M834 all from Dako Ltd, Glostrup Denmark). For antigen retrieval, sections to be stained for eosinophils were pretreated with an aqueous solution of 0.1% trypsin in 0.1% calcium chloride (pH 7.8) at 37° C for 15 minutes. Sections to be stained for macrophages, mast cells and $CD4^+$ T-lymphocytes were pre-treated in microwave oven according to manufacturer instructions. Monoclonal antibody binding was detected with the EXPOSE Mouse and Rabbit Specific AP (red) Detection IHC Kit (AbCam, Cambridge UK). Negative controls, obtained by omission of the primary antibody, revealed no signal. Digital images from the stained sections were obtained with a light microscope (Leica DM 2000) connected to a video recorder and a computerized image analysis system (LAS, Leica Application Suite). In each patient, minimal submucosal area evaluated had to be 0.1 mm^2. Positively stained cells were expressed as the number of cells per mm^2 of examined area.

Statistical Analysis

All cases were coded and the measurements were made without knowledge of clinical data to avoid observer bias. All measurements obtained at baseline and follow-up were used in the data analysis. Children characteristics were summarized using mean±SD or median [range] for continuous variables and counts and percentages for categorical variables. For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Comparisons among groups were evaluated with either Student's t-tests or Mann-Whitney-U test as appropriate. Distributions of categorical variables were compared with the $\chi 2$-test or with Fisher's exact test, when sample size was small ($n< 5$). Correlation coefficients were calculated using the nonparametric Spearman's rank method.

Univariate logistic analyses, followed by a multivariate logistic regression, were performed to detect the strongest predictors of asthma at follow-up. The covariates included in the final models were those that were significantly different between children with and without asthma

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at follow-up in univariate analyses. Values of tissue eosinophils, which were not normally distributed, were log-transformed for logistic analyses. Variables included in the multivariate logistic regression analyses had to be significant in the respective univariate analyses. The collinearity among covariates was estimated with the variance inflation factor (VIF). A VIF ≥ 2 was considered an exclusion criterion. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for all the variables entered in the final model. All analyses were performed using R (version x64 3.3.3 for Windows). All statistical tests were two-tailed and statistical significance was assumed for a p value <0.05.

Results

At baseline, a respiratory paediatrician collected a detailed clinical history, visited the child and administered parental interviews focused on the presence of respiratory symptoms, the treatment during the previous 12 months and the presence of allergic manifestations (Table E1). Moreover, at baseline all children underwent routine blood tests, including complete blood count, total (paper radioimmunosorbent test [PRIST]) and specific (radioallergosorbent test [RAST]) IgE to define the presence of atopy. The presence of atopy was defined by an increase in total (above age-related normal values) and specific IgE (>0.35 kU/L). In particular, specific IgE for the most common aeroallergens were investigated. These included: house dust mite (Dermatophagoides pteronyssinus, Dermatophagoides farinae), moulds (Alternaria alternate), cat dander, grass pollens (Lolium perenne, Poa pratensis; Phleum pratense, Dactylis glomerata, Cynodon dactylon), trees (birch, olive, hazel, cypress), herbs (Artemisiifolia, Artemisia absenthyum, Parietaria judaica), as well as ovalbumin, α and β lactoglobulin. Thirty-five children were atopic, of these 15 were sensitized to one or two allergens and 20 were multi sensitized. Specific IgE results and additional informations on atopy are reported in Table E2.

Table E1. Additional clinical characteristics at baseline.

	Whole cohort	Asthma at follow-up	No asthma at follow-up	*p*
Subjects, n	74	31 (42%)	43 (58%)	
In treatment with ICS, n (%)	17 (23%)	13 (42%)	4 (9%)	<0.0001
In treatment with OCS, n (%)	4 (5%)	2 (6%)	2 (5%)	n.s.
Familiarity for atopy, n (%)	46 (62%)	20 (64%)	26 (60%)	n.s.
Atopic dermatitis, n (%)	7 (9%)	4 (13%)	3 (7%)	n.s.
Allergic rhinitis, n (%)	12 (21%)	5 (16%)	7 (16%)	n.s.

Data on allergic rhinitis are available for 56 out of 74 children

Table E2. Specific allergen sensitization and specific IgE levels (RAST) at baseline according to asthma status at follow-up.

	Sensitized subjects at baseline *n (% of atopics)*	**Specific IgE at baseline** *(kU/L)*	**Asthma at follow up** *n (% of specific sensitized)*
D. pteronyssinus	13 (36%)	31 ± 43	10 (77%)
D. farinae	10 (28%)	40 ± 40	7 (70%)
Alternaria alternate	7 (19%)	9 ± 9.7	4 (57%)
Cat dander	3 (8%)	8.4 ± 8.7	2 (67%)
Lolium perenne	10 (28%)	26 ± 39	5 (50%)
Poa pratensis	1 (3%)	2.6	1
Phleum pratense	4 (11%)	33 ± 46	2 (50%)
Dactylis glomerata	5 (14%)	50 ± 47	1 (20%)
Cynodon dactylon	4 (11%)	30 ± 46	1 (25%)
Olive	3 (8%)	27 ± 46	2 (67%)
Birch	1 (3%)	59	0
Hanzel	1 (3%)	81	0
Cypress	1 (3%)	43	0
Artemisiifolia	2 (6%)	0.5 ± 0.1	1 (50%)
Artemisia absentium	2 (6%)	25 ± 43	0 (0%)
Parietaria judaica	1 (3%)	53	0
OVA	8 (22%)	1 ± 1.4	2 (25%)
α-lactoglobulin	7 (19%)	0.7 ± 0.5	1 (14%)
β-lactoglobulin	7 (19%)	0.7 ± 0.5	3 (43%)

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Figure E1 shows basement membrane (BM) thickness and eosinophils in bronchial biopsies when our cohort was stratified according to the presence (n=35) or absence (n=39) of atopy. We found that BM thickness was associated with asthma development particularly in non-atopic children (panel A), while airway eosinophilia was associated with asthma development in atopic children (panel B). Bottom and top of the box-plot: 25th and 75th percentiles; solid line: median; brackets: 10th and 90th percentiles.

Figure E1

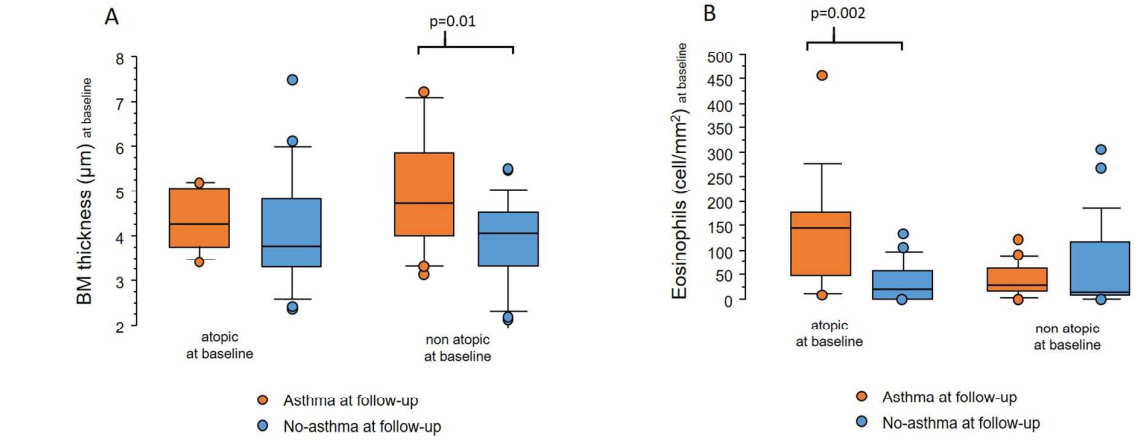

Table E3. Pathological characteristics at baseline and asthma status at follow-up: age stratification considering only children ≤3 yrs at baseline.

	Asthma at follow-up	No asthma at follow-up	*p*
Number of subjects, n	12	12	
Epithelial loss (%)	70 [32 – 100]	83 [20 – 100]	n.s.
BM thickness (μm)	4.7 [3.3 – 100]	3.7 [2.1 – 5.5]	0.03
Eosinophils (cell/mm^2)	28 [11 – 161]	9.5 [0 – 304]	n.s.
Neutrophils (cell/mm^2)	160 [52 – 1023]	217 [46 – 875]	n.s.
Mast-cells (cell/mm^2)	171 [28 – 950]	280 [84 – 650]	n.s.
Macrophages (cell/mm^2)	144 [0 – 597]	80 [0 – 303]	n.s.
CD4$^+$Lymphocytes (cell/mm^2)	135 [0 – 690]	202 [150 – 689]	n.s.

Table E4. Pathological characteristics according to wheezing pattern at baseline.

	Episodic wheezing at baseline	Multitrigger wheezing at baseline	No wheezing at baseline	*p*
Subjects, n	11	30	39	
Epithelial loss (%) [n=80/80]	92 [27 – 100] ¶‡	55 [12 – 100]	43.7 [0 – 100]	0.008
BM thickness (μm) [n=80/80]	3.9 [3.2 – 8]	4.9 [3.3 – 7.4]*	3.7 [2.1 – 4.9]	< 0.0001
Eosinophils (cell/mm^2) [n=73/80]	50 [0 – 455]	60 [0 – 266] ‡	12 [0 – 304]	0.02
Neutrophils (cell/mm^2) [n=74/80]	150 [40 – 580]	244 [0 – 1023]	139 [0 – 925]	n.s.
Mast-cells (cell/mm^2) [n=68/80]	321 [78 – 800] †	324 [0 – 650] †	104 [28 – 950]	0.006
Macrophages (cell/mm^2) [n=71/80]	46 [0 - 457]	115 [0 - 597]	94 [0 - 467]	n.s.
CD4$^+$Lymphocytes (cell/mm^2) [n=69/80]	54.5 [0 -981]	216 [0 -1014]	256 [0 -1108]	n.s.

*significantly different from no wheezing:*p < 0.0001;
† significantly different from no wheezing: †p < 0.05; ‡p < 0.01;
¶ significantly different form multitrigger wheezing: p=0.03

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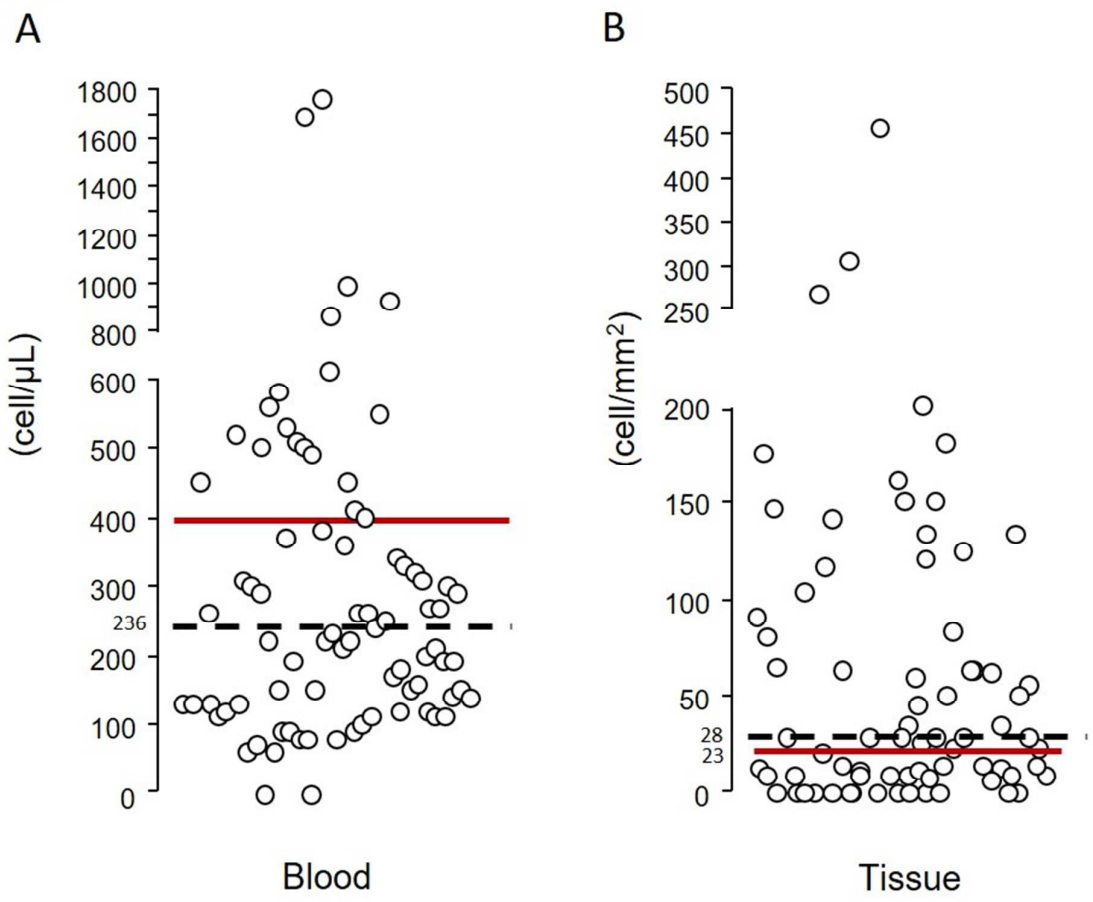
Figure E2 shows eosinophils number in peripheral blood (panel A) and in bronchial biopsies (panel B) in the whole cohort at baseline. Red lines represent the normal limit value (400 cell/μL for blood and 23 cell/mm^2 for tissue), dash lines represent the median value.

Figure E2



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