ORIGINAL RESEARCH

Clinical and Pathologic Factors Predicting Future Asthma in Wheezing Children

A Longitudinal Study

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Abstract

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. In this work, we sought to assess the clinical and pathological factors that might predict the future development of asthma in children. Eighty children (mean age 3.8 ± 1 yr) who underwent a clinically indicated bronchoscopy were followed prospectively for a median of 5 years. At baseline, clinical characteristics with a particular focus on wheezing and its presentation (episodic or multitrigger) were collected, and structural and inflammatory changes were quantified in bronchial biopsies. Follow-up data were available for 74 of the 80 children. Children who presented with multitrigger wheeze were more likely to have asthma at follow-up than those with episodic wheeze ($P = 0.04$) or without wheeze ($P < 0.0001$). Children with asthma also had lower birth weights ($P = 0.02$), a lower prevalence of breastfeeding ($P = 0.02$), 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. We conclude that 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 factor for future asthma.

Keywords: asthma outcome; basement membrane; birth weight; multitrigger/episodic wheezing; preschool wheeze

Clinical Relevance

Clinical and pathological factors that could predict the future development of asthma in wheezing children have not been clearly identified. 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 that is closely associated with the development of asthma, highlighting the importance of airway remodeling in early life as a risk factor for future asthma development.

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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, sex, genetic background, and environmental exposure, the natural history of the disease is still poorly understood. The current knowledge about the 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 3; however, recurrent wheeze in early childhood is not always asthma, and 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 would be to conduct a prospective study of wheezing children to determine whether wheezing (and, if so, which kind of wheezing—multitrigger or episodic) can 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. Although the pathology of asthma is well established, the airway pathology in wheezing children and its potential to predict future development of asthma are less known.

Our aim in this study was to investigate the clinical characteristics (with a particular focus on the presence and type of wheezing) and 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 (mean age 3.8 yr) 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 the Department of Women's and Children's Health, University of Padova, Italy, from 2002 to 2014. All of the children underwent bronchoscopy for the appropriate clinical indications according to European Respiratory Society guidelines (11), as summarized in Table 1. Fiberoptic bronchoscopy was well tolerated by all of the children.

Respiratory symptoms (particularly wheezing and its pattern [multitrigger or episodic]) were diagnosed at baseline by a respiratory pediatrician. 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 in the data supplement).

Episodic wheeze and multitrigger wheeze were defined according to the European Respiratory Society 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, whereas multitrigger wheeze is wheezing that shows discrete exacerbations, but also symptoms between episodes (12). The severity of wheezing was graded on a scale from 0 to 3 (0: no symptoms; 1: mild; 2: moderate; 3: severe), and the frequency of wheezing was graded on a scale from 0 to 6 (0: no episodes; 1: less than one episode/month; 2: one episode/month; 3: two to three episodes/month; 4: one episode/week; 5: more than one episode/week; 6: daily episodes).

At baseline, all children underwent routine blood tests, including complete blood cell counts and total and specific IgE levels The presence of atopy was defined by an increase in total and specific IgE levels (IMMunoCAp; Phadia) (Table E2 in the data supplement). Spirometry was performed only in children who were able to cooperate with the test.

At the follow-up visit, a respiratory pediatrician interviewed the children's parents or the study subjects and conducted a detailed clinical investigation to confirm or exclude asthma diagnosis. The asthma diagnosis was obtained by the respiratory pediatrician 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 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 the follow-up visit, the typical symptoms (wheezing, shortness of breath, and cough) were to be associated with at least one of the following conditions: *1*) treatment with regular or as-needed asthma medications, and *2*) the presence of airflow obstruction that was reversible with bronchodilators. At the follow-up visit, pulmonary function tests (Superspiro; Micro Medical) and fractional exhaled nitric oxide ($F_{E_{NO}}$) measurements (NIOX VERO; Aerocrine) were performed.

Full details regarding the bronchoscopy and bronchial biopsy procedures have been previously described (14, 15). Briefly, the biopsies were formalin fixed and paraffin

Table 1. Clinical Indications for Bronchoscopy*

	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 pneumonia	1 (1%)	0	1 (2%)

*According to European Respiratory Society Guidelines (Reference 11).
†Six out of 80 children were lost during the follow-up.

embedded, and then 5-μm-thick sections were stained with hematoxylin-and-eosin to quantify epithelial loss and basement membrane (BM) thickness. Immunohistochemical techniques were used to quantify inflammatory cells (eosinophils, neutrophils, mast cells, $CD4^+$ T lymphocytes, and macrophages; *see* the data supplement). To avoid observer bias, all cases were coded and measurements made without knowledge of the clinical data. Coefficients of variation for repeated measurements reflecting inter- and intraobserver variability ranged from 4% to 7% for epithelial loss and BM thickness, and from 5% to 10% for inflammatory cells. Written consent was obtained from the 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

The children's characteristics were expressed using the 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* test or the Mann-Whitney *U* test as appropriate. Distributions of categorical variables were compared using the χ^2 test or Fisher's exact test when the 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 ×64 3.3.3 for Windows) as detailed in the data supplement. 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 ≤5 years of age (mean 3.8 ± 1 yr). The median follow-up duration was 5 years (range 1–13 yr). Figure 1 summarizes the outcomes of the study. Follow-up data were available for 74 children: 54 out of 80 attended a follow-up visit, and 20 children who lived far away from our hospital completed a questionnaire by telephone and provided available clinical records. The clinical characteristics at baseline of the six children lost at follow-up were not different from those of the remaining 74. At baseline, 38% of the children had multitrigger wheezing, 15% had episodic wheezing, and 47% had no wheezing.

Table 2 illustrates the clinical characteristics of all of the children at baseline according to their asthma status at follow-up. At follow-up, 31 children in our cohort had confirmed asthma (42%), and 43 did not (58%). Children with asthma at follow-up did not differ from those without asthma in sex distribution, age at baseline, age at symptom onset, and follow-up duration. Subjects who had asthma at follow-up were more likely to have wheezing at baseline ($P = 0.0002$). When the pattern of wheezing (multitrigger or episodic) was examined, it was found that children with multitrigger wheezing at baseline were more likely to have asthma at follow-up (71%) than children with episodic wheezing (36%) or without wheezing (20%) ($P = 0.04$, $P < 0.0001$; Figure 1). Both the 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 with children who did not. Similarly, when we analyzed separately children with only one or two 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 multisensitized children (>2 allergens; 53%) than in children with 2 or less sensitizations (35%) but the difference was not significant. Of interest, children with asthma at follow-up had lower birth weight and less breastfeeding than those without asthma (both $P = 0.02$). The two groups did not differ with regard to pulmonary

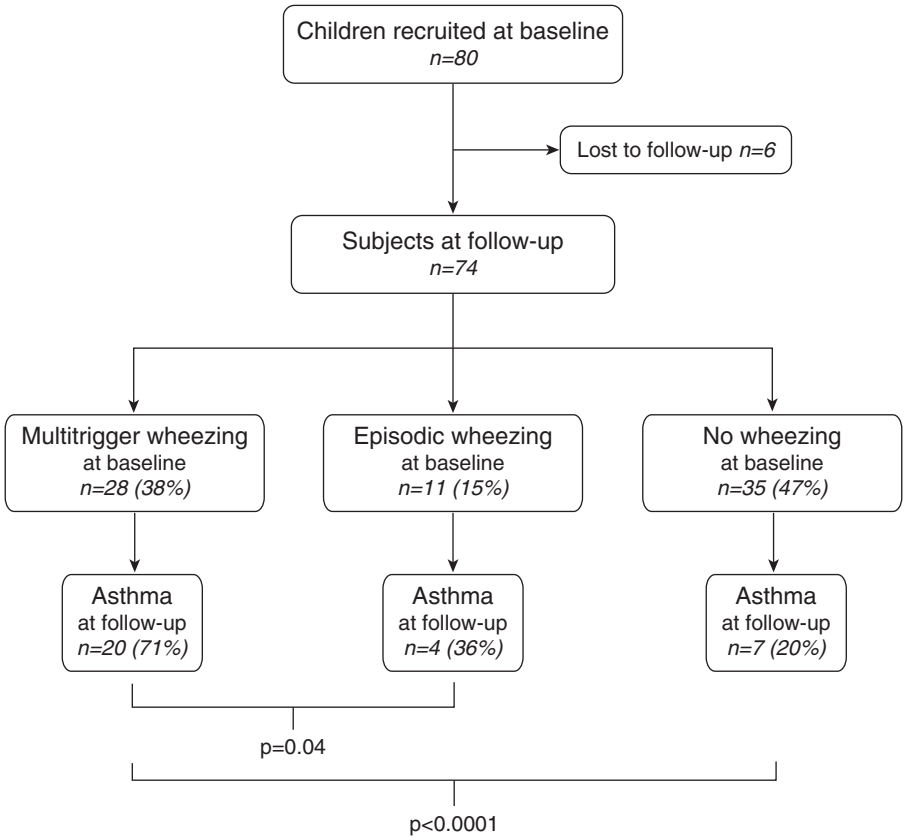

Figure 1. Flow diagram of the children included in our study stratified according to the presence and type of wheezing (multitrigger/episodic/no wheeze) at baseline. The overall attendance rate was 92.5%.

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%)	
Sex, male (%)	38 (51%)	16 (51%)	22 (51%)	n.s.
Age, yr	3.8 ± 1	3.7 ± 1	3.8 ± 0.9	n.s.
Age symptom onset, yr	1.6 ± 1.13	1.5 ± 1.1	1.9 ± 1.1	n.s.
Follow-up duration, yr	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	28 (38%)	20 (83%)	8 (53%)	0.04
Episodic	11 (15%)	4 (14%)	7 (47%)	
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, % predicted*	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 (0–3,647)	69 (0–3,647)	38 (0–2,188)	0.07
Blood eosinophils, cells/μl	236 (0–1,760)	310 (0–1,760)	210 (0–990)	n.s.
Birth weight, g†	3,386 ± 661	3,092 ± 631	3,542 ± 632	0.02
Bronchiolitis, *n* (%)‡	19 (32%)	7 (32%)	12 (32%)	n.s.
Breastfeeding > 3 mo, *n* (%)§	32 (60%)	7 (39%)	25 (71%)	0.02
Parental smoking, *n* (%)‖	18 (45%)	7 (47%)	11 (44%)	n.s.

Definition of abbreviations: FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; n.s. = not significant.
Data are expressed as counts (percentages); mean ± SD or median (range). *P* values refer to the comparison between children with asthma and those with no asthma at follow-up.
*Data at baseline were available for a subset of children (17/74).
†Data at baseline were available for a subset of children (46/74).
‡Data at baseline were available for a subset of children (59/74).
§Data at baseline were available for a subset of children (53/74).
‖Data at baseline were available for a subset of children (40/74).

function parameters, blood eosinophils, history of previous bronchiolitis, or 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 in the data supplement).

Figure 2 illustrates the distribution of body weights at birth, showing that children who developed asthma at follow-up had lower birth weights than those who did not, even if the median birth weight values were in the physiological range (Figure 2A). When stratified by the type of wheezing at baseline (Figure 2B), 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 (Figure 2B).

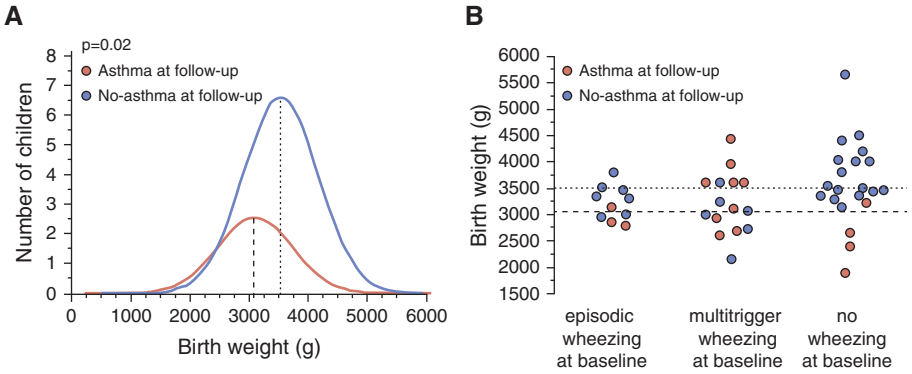

Figure 2. (*A*) Frequency distribution curves for the values of birth weight in children with asthma (orange) and without asthma (blue) at follow-up. (*B*) Scatterplot reporting birth weights in children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze). The dashed line represents the median birth weight value for children with asthma, and the dotted line represents the median birth weight value for children without asthma. Data for this analysis were available for 46 out of 74 children.

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. Ages at follow-up were similar between children who developed asthma (8.9 ± 2.9 yr) and those who did not (9.5 ± 2.7 yr). As expected, subjects with asthma at follow-up had increased frequency of wheezing and increased use of as-needed bronchodilators compared with those who did not develop asthma. Overall, asthma was well controlled in the majority of subjects with asthma at follow-up: 77% were treated with inhaled corticosteroids and used as-needed bronchodilators a median of twice per month. Only one subject had severe asthma and needed treatment with oral steroids. Of note, subjects with asthma were prescribed more courses of antibiotics and had more lower-respiratory-tract infections (bronchitis and 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 subjects with and without asthma at follow-up. Of note, children with asthma at follow-up had significantly increased Fe_{NO} values compared with those without asthma ($P = 0.04$). When we performed a Fe_{NO} analysis in children with asthma using the previously reported cutoff of 20 ppb (16, 17), we found that 47% of the children with asthma had $Fe_{NO} > 20$ ppb, and 53% had $Fe_{NO} < 20$ ppb. When we compared children with asthma and $Fe_{NO} > 20$ ppb with those with $Fe_{NO} < 20$ ppb, we observed no differences in symptoms, FEV_1, FEV_1/FVC, or 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

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, yr	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, % predicted*	93 ± 16	93 ± 19	93 ± 14	n.s.
FEV_1/FVC, %*	86 ± 7	83 ± 8	88 ± 7	n.s.
$F_{E_{NO}}$, ppm†	20 ± 14	27 ± 18	16 ± 11	0.04

Definition of abbreviations: AB = antibiotics; BD = bronchodilator; $F_{E_{NO}}$ = fractional exhaled nitric oxide; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; ICS = inhaled corticosteroids; LRI = lower respiratory infection; n.s. = not significant; ppm = parts per million.
Data are expressed as counts (percentages); mean ± SD or median (range). *P* values refer to the comparison between children with asthma and those with no asthma at follow-up.
*Data were available for a subset of children (53/74).
†Data were available for a subset of children (49/74).

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 with those who did not. The percentage of damaged epithelium and the number of neutrophils, macrophages, mast cells, and $CD4^+$ lymphocytes in bronchial biopsies did not differ between the two groups of children, and neither did inflammatory cells and mediators in BAL (Table 4). Figure 3 illustrates the distribution of BM thicknesses at baseline, showing that children who developed asthma at follow-up had thicker BMs than those who did not (Figures 3A and 3B). This finding was confirmed even when only children who were ≤3 years of age were considered ($n = 24$; Table E3). Children with a history of wheezing (mainly multitrigger wheezing) at baseline had significantly thicker BMs than nonwheezing children (Figure 3C). BM thickness was positively correlated with the frequency ($r = 0.49$; $P < 0.0001$) and severity of wheezing ($r = 0.4$; $P = 0.0005$) at baseline, and with the frequency of wheezing ($r = 0.41$; $P = 0.0005$) and use of bronchodilators ($r = 0.26$; $P = 0.03$) at follow-up. Because 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 data supplement.

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, cells/mm^2 (*n* = 68/74)	61 (0–455)	14 (0–304)	0.026
Neutrophils, cells/mm^2 (*n* = 70/74)	120 (0–1,023)	197 (0–925)	n.s.
Mast cells, cells/mm^2 (*n* = 63/74)	271 (28–950)	144 (0–800)	n.s.
Macrophages, cells/mm^2 (*n* = 67/74)	92 (0–597)	118 (0–467)	n.s.
$CD4^+$ lymphocytes, cells/mm^2 (*n* = 67/74)	312 (0–1,014)	200 (0–1,108)	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 (2–3,000)	306 (36–5,728)	n.s.

Definition of abbreviations: BM = basement membrane; ECP = eosinophilic cationic protein; n.s. = not significant.
Data are expressed as median (range).

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 (Figure 4A). 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 with nonwheezing children (Figure 4B). Of interest, children with episodic wheezing at baseline had increased epithelial damage and increased numbers of mast cells in bronchial biopsies compared with nonwheezing children (Table E4).

When we examined the relationship between the number of eosinophils in airway tissue and the number of eosinophils in blood, we found a weak, albeit significant, correlation ($r = 0.24$, corresponding to $r^2 = 0.06$), indicating that blood eosinophils cannot be considered representative of tissue eosinophils in our population (Figure 5). No correlations between blood and tissue eosinophils were observed when only values above or below the normal limits (18) were considered (Figure E1 in the data supplement).

A subset of children included in our study ($n = 17$; 23%) were 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 who were not treated with inhaled steroids ($n = 57$), the main findings were confirmed (baseline BM thickness and airway eosinophilia were increased in subjects with asthma at follow-up; $P < 0.05$ for both).

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

Discussion

Wheezing in early childhood is a common worrying event for families and

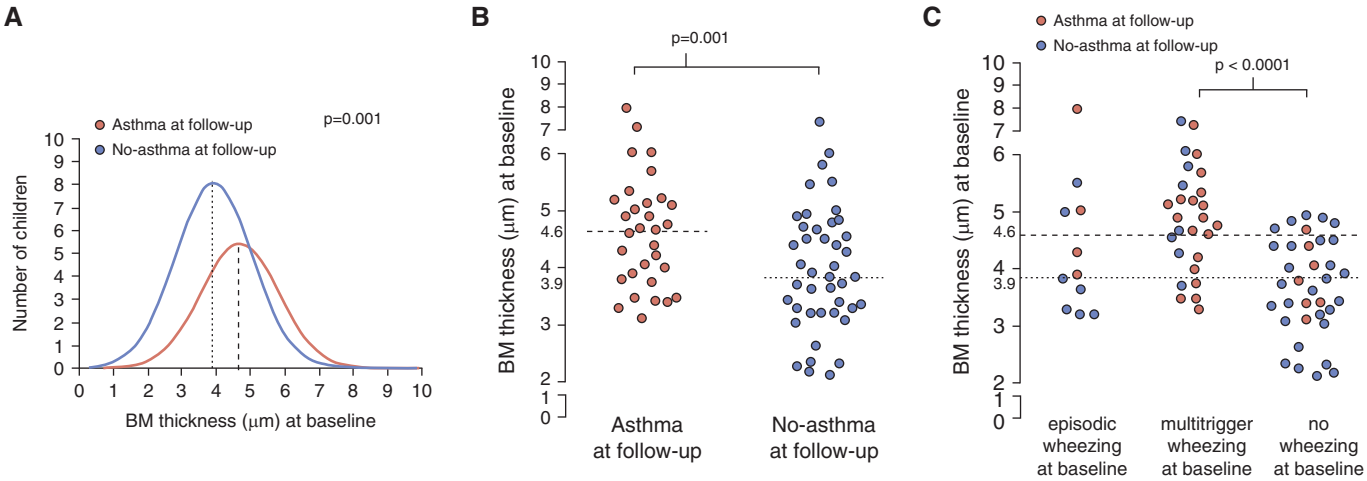

Figure 3. (*A*) Frequency distribution curves for the values of basement membrane (BM) thickness in children with asthma (orange) and without asthma (blue) at follow-up. (*B*) Scatterplot reporting BM thickness in children with asthma (orange) and without asthma (blue) at follow-up. (*C*) Scatterplot reporting 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 children with asthma, and the dotted line represents the median BM value for children without asthma. Data for this analysis were available for all children (n = 74).

pediatricians alike, because it might herald the development of asthma. However, there is the possibility that the wheezing is 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 undergone bronchial biopsies, and assessed their clinical characteristics (with a particular focus on wheezing), airway pathology, and eventual asthma development. Our results showed that multitrigger wheezing, BM thickening, and reduced birth weight were significantly associated with the development of asthma at follow-up.

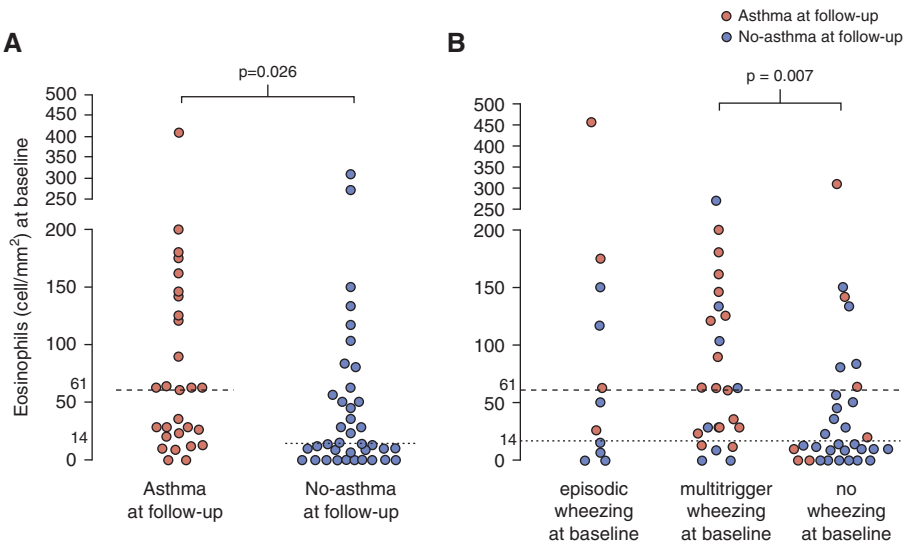

Figure 4. (*A*) Scatterplot reporting eosinophils in bronchial biopsies from children with asthma (orange) or without asthma (blue) at follow-up. (*B*) Scatterplot reporting eosinophils in bronchial biopsies from children of our cohort stratified according to the presence and type of wheezing at baseline (multitrigger/episodic/no wheeze). The dashed line represents the median eosinophil value for children with asthma, and the dotted line represents the median eosinophil value for children without asthma. Data for this analysis were available for 68 out of 74 children.

We defined the wheezing pattern in our cohort using the two symptom-based phenotypes of wheeze: episodic (triggered mostly by viral respiratory infections, with affected children being symptom-free between episodes) and multitrigger (triggered by viruses and other causes such as allergens, characterized by the presence of symptoms between episodes) (12). Once we had defined the wheezing pattern and obtained bronchial biopsies, we followed our cohort for a median of 5 years after the first evaluation, and found that 71% of the multitrigger wheezers had asthma at follow-up, whereas only 36% of the episodic wheezers did. 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, whereas 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 two large longitudinal cohorts suggests that multitrigger and, to a lesser extent, episodic wheeze tend to persist longitudinally regardless of wheezing

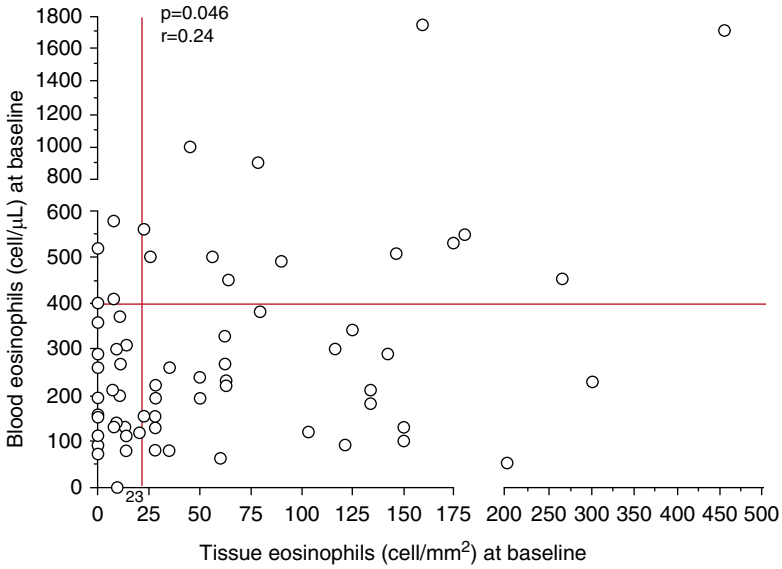

Figure 5. Relationship between eosinophils in blood and eosinophils in tissue (bronchial biopsies) at baseline in all of the children in the cohort. Spearman's rank correlation coefficient $P = 0.046$; $r = 0.24$ ($r^2 = 0.06$). The red line represents the cutoff for normal values of eosinophils in tissue (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.

severity (23). Our results, which were based on clinical information (including both the type and severity of wheezing) that was carefully collected during a single visit, demonstrate that wheezing information can be valuable and predictive of the future development of asthma.

Among the other clinical factors examined, we observed that low birth weight and reduced breastfeeding 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 (~500 g lower) and significantly less breastfeeding 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 to the mechanism of asthma development, especially in children with episodic wheeze or no wheeze (Figure 2). The mechanisms underlying the association between low birth weight and asthma outcomes are not completely 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 develop asthma. This confirms the role of atopy as a predictor of asthma persistence throughout childhood and adolescence (3, 29), directly through early allergen sensitization and indirectly by impairing the immune response to viral infections (30, 31).

To determine whether wheezing and asthma development at follow-up were linked to airway pathology at baseline, we prospectively investigated children who had to undergo diagnostic bronchoscopies. All of the children in our cohort, who had a mean age of 3.8 years, had bronchoscopies performed for the appropriate clinical indications, according to international guidelines (11). The bronchial biopsies (obtained with approval of the local ethics committee and with consent) provided a pathological basis for the clinical and wheezing characteristics and their association with future asthma outcomes.

BM thickening at baseline was significantly increased in children who developed asthma at follow-up compared with children who did not. Importantly, when our cohort was stratified by age, BM thickness was the only factor that was associated with asthma at follow-up in children who were ≤3 years of age at baseline. The multivariate analysis of our data showed that BM 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 children with asthma were also performed, but BM thickening was not found to predict asthma development. These differences may be due to the dissimilar populations studied. The children in those studies were younger, had severe airway obstruction, and had all been treated with high-dose corticosteroids at the time of bronchoscopy (32–34). By contrast, the

Table 5. Logistic Regression Analysis in Relation to Asthma at Follow-up

	Univariate Logistic Regression Analysis			Multivariate Logistic Regression Analysis		
	OR	95% CI	*P* Value	OR	95% CI	*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, <3,000 *g*	6.5	1.5–27.4	0.01	10.3	1.5–27.5	0.01
Breastfeeding, >3 mo	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, cells/mm^2	2.3	1.1–4.6	0.02	1.6	0.9–3.1	n.s.

Definition of abbreviations: BM = basement membrane; CI = confidence interval; OR = odds ratio; n.s. = not significant.

children in our study were older, their asthma was not severe, and most had not been treated with corticosteroids at the time of bronchoscopy. When we reanalyzed our data excluding the few children who were on corticosteroids, our results did not change.

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 wheezing and episodic wheezing are truly different conditions, with different pathogenetic mechanisms, or simply different severity classes of the same underlying conditions (21, 37–39). Our results from a similar pathology substrate in the two forms of wheezing support the latter conclusion.

A possible limitation of our study is that the cohort of children who underwent a clinically indicated bronchoscopy may not be representative of wheezing children in general, as the concomitant diseases could have influenced the results. However, these concomitant conditions were evenly distributed among the 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 remodeling 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 BM, and can be more easily sampled by endobronchial biopsies (40).

It should be acknowledged that even if the differences in BM thickness ($P = 0.001$) and tissue eosinophils ($P = 0.026$) between children with asthma and those without asthma were statistically significant, there was a considerable overlap at the individual level between the two groups (Figures 3B and 4A), as can be expected for any credible biological measurements. Therefore, these measurements should not be used as predictors at the individual level, but they can be of pathological importance in comparisons between groups, and can help to further elucidate the early mechanisms of asthma. Finally, a potential limitation of this study is that we did not perform any mechanistic analyses of BAL, as 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 BM in early childhood is a pathological finding that is closely associated with the development of asthma, highlighting the importance of airway remodeling in early life as a risk factor for future asthma development. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Online Data Supplement

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

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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

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).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%)

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.

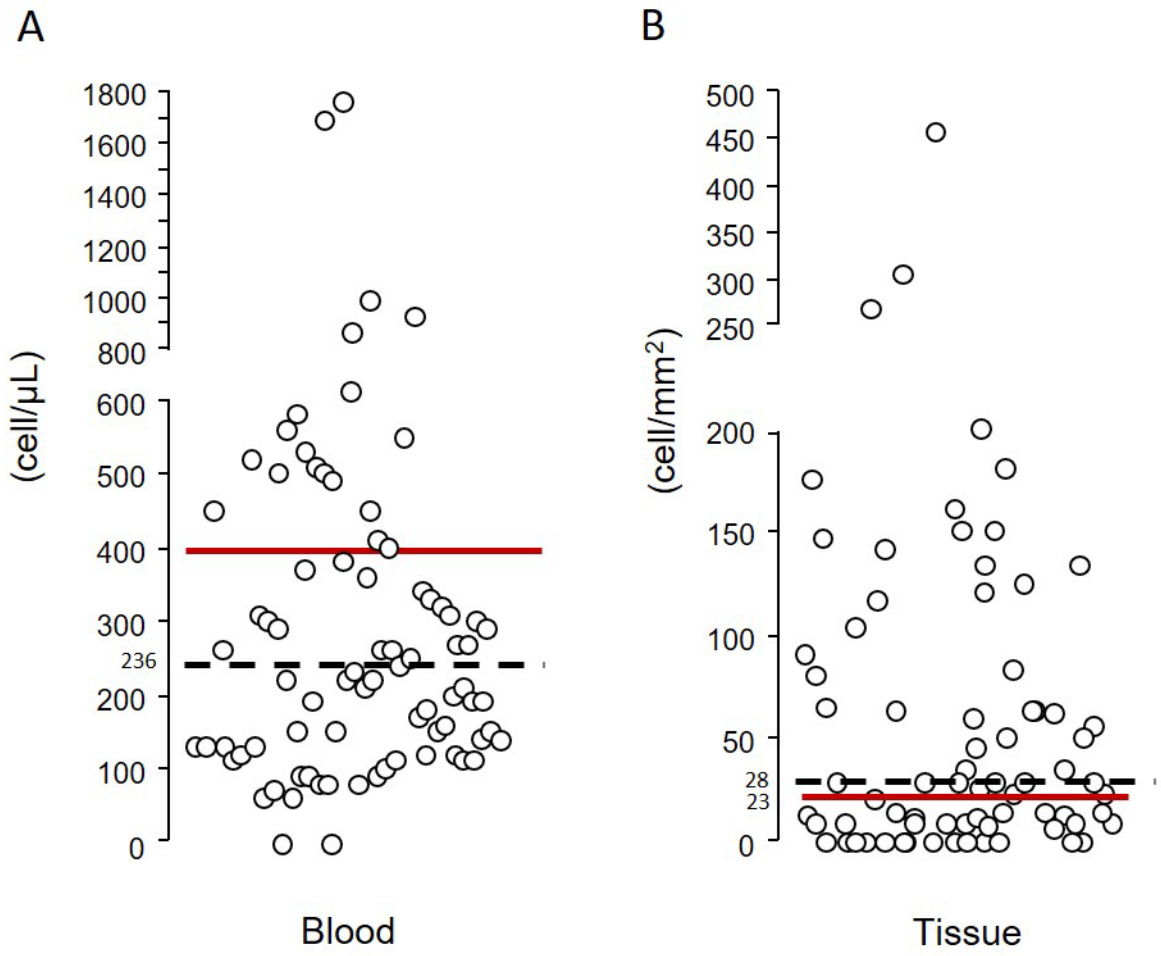
Table E4. Pathological characteristics according to wheezing pattern at baseline.

	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

Figure E1 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 E1



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