

Early Detection of Airway Wall Remodeling and Eosinophilic Inflammation in Preschool Wheezers

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Rationale: It is unclear when the pathologic features of asthma first appear. We hypothesized that eosinophilic airway inflammation and epithelial reticular basement membrane (RBM) thickening, absent in wheezy infants, would be present in preschool children with severe, recurrent wheeze.

Objectives: To compare RBM thickness and inflammation in endobronchial biopsies (EBs) from wheezy preschool children and age-matched control subjects.

Methods: EBs were obtained from wheezy preschool children (aged 3 mo to 5 yr), undergoing a clinically indicated fiberoptic bronchoscopy. Subjects undergoing fiberoptic bronchoscopy to investigate stridor acted as nonasthmatic controls. RBM thickness was measured and the density of subepithelial, immunologically distinct inflammatory cells was determined and expressed as a volume fraction (%). EBs from 16 children (median age, 29 [7–57] mo) with wheeze confirmed by video questionnaire (confirmed wheezers [CWs]), 14 with reported wheeze (reported wheezers [RWs]) (median age, 17 [8–58] mo), and 10 control subjects (median age, 19 [5–42] mo) were assessed.

Measurements and Main Results: RBM thickness in the three groups was as follows: CWs: median, 4.6 (range, 2.9–8.0) μ m; RWs: median, 3.5 (2.1–5.4) μ m; control subjects: median, 3.8 (2.5–4.7) μ m. RBM was significantly thicker in CWs than in control subjects ($P < 0.05$). Eosinophil density was as follows: CWs: median, 1.07% (range, 0.0–3.52%); RWs: median, 0.72% (0.0–2.04%); control subjects: median, 0.0% (0.0–1.05%). Eosinophilic inflammation was significantly greater in CWs compared with control subjects ($P < 0.05$). There were no between-group differences for any other inflammatory cell phenotype.

Conclusions: The characteristic pathologic features of asthma in adults and school-aged children develop in preschool children with confirmed wheeze between the ages of 1 and 3 years, a time when intervention may modify the natural history of asthma.

Keywords: asthma; pediatrics; pathology

Thickening of the bronchial epithelial reticular basement membrane (RBM) and eosinophilic airway inflammation are characteristic pathologic features of asthma in adults (1). The former has also been reported in school-aged children with asthma (2–5), and more recently in occasional cases of children with asthma as young as 3 years old (6). Tissue eosinophilia and features of airway remodeling, including increased epithelial loss and subepithelial vessels, have also been reported in children, median

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Eosinophilic inflammation and reticular basement membrane thickening, the pathologic hallmarks of asthma, are not present in infant wheezers but are seen in schoolchildren with asthma.

What This Study Adds to the Field

The reticular basement membrane thickening and eosinophilic inflammation seen in adults are present in wheezy preschool children.

age 5 years (range, 2–15 yr), with mild to moderate asthma (6). However, we have shown that bronchial RBM thickening and eosinophilic inflammation are absent in wheezy infants (median age, 12 mo) with reversible airflow obstruction (7). Because there are no reports on airway pathology in preschool wheezers alone, we investigated bronchial tissue in this group as this is a time when the changes characteristic of adult asthma are likely to begin.

Martinez has suggested that “the greatest obstacle to the development of approaches for the prevention of asthma has been the lack of a clear understanding of its natural history” (8). Most preschool children with wheeze lose their symptoms by the time they reach school age, whereas only a minority remain symptomatic, develop persistent wheeze, and are subsequently diagnosed with asthma. Their lung function, presumed normal at birth (9), is reduced by age 3 years (10) and remains so at both 6 (11) and 16 years (9). The pathologic counterparts of the early lung function changes have never been described in the preschool age group and it is not known when they first appear. Moreover, there is a need to identify those preschool wheezers at high risk of developing asthma and to initiate effective preventive measures at the appropriate early time (8). We have already shown that even atopic wheezers with documented bronchodilator reversibility have no evidence of increased airway wall inflammation or RBM thickness at a median age of 12 months, thus narrowing the time window during which these changes develop. Thus, we hypothesized that RBM thickening is present and associated with eosinophilic inflammation in preschool children with severe, recurrent wheeze at approximately 3 years of age, corresponding to the first reported decrements in lung function (10). Some of the results reported herein have been reported previously in abstract form (12).

METHODS

Subjects

The study was approved by the Brompton, Harefield, and the National Heart and Lung Institute Ethics Committee. Children aged between

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3 months and 5 years referred for further investigation of severe, recurrent wheeze and due to undergo a detailed protocol of investigations, including fiberoptic bronchoscopy (FOB), were eligible for recruitment (13). All had recurrent, noisy breathing, interpreted as wheeze, either when assessed during acute symptoms or from parental reports. Each had at least three such episodes lasting more than 3 days in the previous 6 months. The following patterns were identified: (1) symptoms present on most days, (2) episodic symptoms only with colds, and (3) acute exacerbations with colds and interval symptoms between colds. *See the online supplement for details.*

Nonwheezing Control Subjects

Nonwheezing control subjects had no history of wheezing or lower respiratory symptoms. The majority were undergoing a clinically indicated bronchoscopy to investigate troublesome stridor, or to assess airway compression from a vascular ring. Two were undergoing a general anesthetic for other reasons and had undergone a research bronchoscopy and biopsies (*see the online supplement*).

At the time of recruitment, informed, written parental consent was obtained to take biopsies for the additional purpose of research.

Video Questionnaire

To establish objectively the presence or absence of wheeze, parents were shown a previously validated video questionnaire (14) of symptomatic infants and preschool children demonstrating wheeze, stridor, and two different upper respiratory noises. Subjects and control subjects were divided into three groups according to answers to the video questionnaire:

1. Confirmed wheezers (CWs): subjects whose parents reported wheeze and identified wheeze from the video questionnaire
2. Reported wheezers (RWs): subjects whose parents reported wheeze, but did not identify wheeze from the video questionnaire
3. Control subjects: subjects whose parents reported stridor or no respiratory symptoms and did not identify wheeze from the video questionnaire (*see the online supplement*)

School-aged Children with Asthma

To provide a comparison of RBM thickness in preschool wheezers, controls, and older children with asthma, endobronchial biopsies (EBs) from 28 school-aged children with difficult asthma, collected in a previous study (15) that had used the same technique for taking and processing biopsies, were reassessed by our lead investigator (S.S.).

Atopic Status

Atopy was defined as the presence of eczema and/or at least one positive specific IgE radioallergosorbent test to the following food or aeroallergens: milk, egg, peanut, house dust mite, cat, dog, and grass pollens.

Bronchoscopy, Biopsy, and Processing

FOB and EB were performed under general anesthesia as previously described (16). *See the online supplement for details on these procedures and biopsy processing and immunostaining.*

Quantification of RBM Thickness and Inflammatory Cells

RBM was measured using computer-aided image analysis as previously described (17). The density of subepithelial immunostained cells was assessed by "point counting" sections at $\times 400$ magnification. The cell's "volume density" was calculated (excluding areas of smooth muscle, vessels, and glands), and expressed as a percentage (18) (*see the online supplement*).

Variability and Statistical Analysis

The average coefficient of variation (CV) percentage for the intra-observer variability of RBM thickness was 7.0% and the CV percentages for assessing the volume densities of CD45⁺ cells and eosinophils (i.e., EG2⁺ cells) were 12 and 8.5%, respectively. Differences between groups were assessed first using the Kruskal-Wallis test, which, if significant, was followed by between-two-group comparisons using the Mann-Whitney U test and a Bonferroni correction for multiple com-

parisons. Spearman rank correlation was used to assess associations (*see the online supplement*).

RESULTS

Bronchoscopies were well tolerated. Twenty-seven CWs, 20 RWs, and 21 control subjects were recruited. Biopsies from 40 of these 68 (59%) preschool children were of sufficient quality to assess both the RBM and subepithelial inflammation. There were 16 CWs (median age, 29 [7–57] mo), 14 RWs (median age, 17 [8–58] mo), and 10 control subjects (median age, 19.5 [5–42] mo). Their clinical characteristics are summarized in Table 1.

RBM Thickness in Wheezers and Control Subjects

RBM thickness in the three groups was as follows: CWs: median, 4.6 (range, 2.9–8.0) μm ; RWs: median, 3.5 (range, 2.1–5.4) μm ; and control subjects: median, 3.8 (range, 2.5–4.7) μm . RBM was significantly thicker in CWs compared with control subjects ($P < 0.05$). There was no significant difference in RBM thickness between CWs and RWs or between RWs and control subjects (Figures 1a and 2).

Age. There was no significant correlation between RBM thickness and age in CWs (Spearman $r^2 = 0.01$, $P = 0.61$) or RWs (Spearman $r^2 = 0.02$, $P = 0.59$). However, there was a positive linear relationship between age and RBM thickness in the control subjects (Spearman $r^2 = 0.31$, $P = 0.02$). The contribution to data by infants (aged 3–24 mo) was excluded in a further *post hoc* analysis. Subjects were subdivided according to age using 24 months as a cutoff. A strong trend for a thicker RBM in CWs older than 24 months compared with age-matched control subjects remained (median RBM thickness: CWs, 5.0 [range, 3.4–8.0] μm , compared with control subjects, 3.76 [3.2–5.5] μm ; $P = 0.058$) (Figure E1 of the online supplement). There was no difference in RBM thickness between CWs and control subjects in subjects younger than 24 months.

Atopy. When all wheezers and control subjects were divided according to atopic status, RBM thickness was similar in atopic and nonatopic children (atopic median, 3.6 [2.0–5.7] vs. nonatopic median, 3.8 [2.5–8.0] μm ; not significant) (Figure E1). There was no difference in RBM thickness between atopic and nonatopic CWs, or atopic and nonatopic control subjects (*see Figure E2*). Figure 1b highlights patients according to wheeze category and atopic status.

Steroid therapy. Ten of 16 (63%) CWs and 7 of 14 (50%) RWs were prescribed inhaled steroids. There was a strong trend for the RBM of CWs receiving inhaled steroids to be thicker than that of CWs not receiving inhaled steroids (CWs receiving steroids, median, 5.5 [3.5–8.0] vs. CWs not receiving steroids, 4.2 [2.9–5.3] μm ; $P = 0.056$). There was no difference in RBM thickness between RWs receiving inhaled steroids and those not receiving inhaled steroids (Figure 1c).

Six of 16 (38%) CWs were taking oral steroids either at the time of the biopsy or had had them been prescribed in the previous 2 weeks. The RBM was significantly thicker in subjects taking oral steroids compared with those not taking oral steroids (median, 6.1 [range, 4.6–8.0] vs. 4.2 [2.9–7.1] μm ; $P < 0.05$).

Viral wheeze. There was no significant difference in RBM thickness when subjects with presumed viral wheeze were compared with control subjects. However, the RBM was significantly thicker in subjects with interval wheeze (wheeze both with and apart from colds) compared with controls (subjects with interval wheeze, median, 4.2 [2.1–8.0] vs. control subjects, 3.3 [2.0–4.7] μm ; $P < 0.01$).

RBM thickness in CWs, control subjects, and school-aged children with asthma. Although the RBM of CWs (median, 4.6

TABLE 1. CLINICAL CHARACTERISTICS OF CONFIRMED WHEEZERS, REPORTED WHEEZERS, AND CONTROL SUBJECTS WITH BIOPSIES OF SUFFICIENT QUALITY TO ASSESS THE RETICULAR BASEMENT MEMBRANE AND SUBEPITHELIAL INFLAMMATION

	Confirmed Wheeze (n = 16)	Reported Wheeze (n = 14)	Controls (n = 10)	P Value
Male/female, n	8/8	7/7	6/4	
Age, mo, median (range)	29 (7–57)	17 (8–58)	19.5 (5–42)	NS
Weight, kg, median (range)	14.1 (7.04–25.2)	11.2 (6.4–19.6)	12.2 (6.92–18.7)	NS
Birth gestation, wk, median (range)	40 (33–42)	40 (34–42)	39 (35–40)	NS
Birth weight, kg, median (range)	3.75 (2.33–4.46)	3.61 (1.6–4.22)	3.1 (2.5–5.03)	NS
Atopic (eczema and/or ≥1 positive RAST), n (%)	11 (68.8)	8 (57.1)	4 (40)	NS
Doctor-diagnosed eczema, n (%)	9 (56.3)	6 (42.9)	3 (30)	NS
≥1 positive RAST, n (%)	11 (68.8)	7 (50)	2 (20)	
Inhaled/oral steroids, n (%)	10 (62.5)	8 (57)	0	
Main symptom, n				
Wheeze	9	7	0	
Wheeze and cough (equal)	4	6	0	
Cough (>wheeze)	3	1	2 (barking)	
Stridor	0	0	4	
None	0	0	3	
Tachypnea	0	0	1	
Video symptom, n				
Wheeze	10	0	0	
Wheeze and URN	6	0	0	
URN	0	8	0	
None	0	6	5	
Stridor	0	0	5	
Previously admitted for wheeze, n	15	12/14	0	
No. of admissions per patient, median (range)	6.5 (0–20)	2 (0–15)	0	<0.05*
Family history, n				
Maternal asthma	2	2	2	
Paternal asthma	3	2	0	
Smoking during pregnancy, n				
Mother alone	3	0	0	
Father alone	1	0	3	
Both parents	2	4	0	
Current smoking, n				
Mother	5	4	2	
Father	3	3	3	
Pets				
Cat	1	2	1	
Dog	0	4	0	
Daycare < 1 yr old, n	2	0	1	

Definition of abbreviations: NS = not significant; RAST = radioallergosorbent test; URN = upper respiratory noise.

* Confirmed wheeze compared with reported wheeze and controls.

[range, 2.9–8.0] μm) was significantly thicker than preschool non-wheezing control subjects (median, 3.8 [range, 2.5–4.7] μm ; $P < 0.05$), it was not as thick in the CWs compared with the school-aged children with difficult asthma (median, 6.7 [range, 4.5–10.0] μm ; $P < 0.01$, CWs vs. children with difficult asthma) (Figure 3).

Subepithelial Inflammation

Inflammation in CWs, RWs, and control subjects. There was no difference between CWs, RWs or between CWs or RWs and the control subjects regarding the density of neutrophil elastase positive (NE⁺), CD4⁺, CD8⁺, or CD45⁺ cells (the last used to assess the total number of leukocytes). The density of EG2⁺ cells in the three groups was as follows: CWs: median, 1.07% (range, 0.0–3.52%); RWs: median, 0.72% (range, 0.0–2.04%); and control subjects: median, 0.0% (range, 0.0–1.05%). Eosinophil density was significantly greater in CWs compared with control subjects ($P < 0.05$). There was no difference in eosinophil density between CWs and RWs, or RWs and control subjects (Figures 2 and 4a).

Atopy. When all wheezers and control subjects were considered together, there was no difference in EG2⁺, NE⁺, CD4⁺, CD8⁺, or CD45⁺ between atopic and nonatopic patients (Figure E3). There was also no difference in eosinophilic in-

flammation between atopic wheezers and nonatopic wheezers, and no difference between atopic and nonatopic control subjects (Figure E4 and Figure 4b).

Eosinophilic inflammation and steroid therapy. CWs receiving inhaled steroids had a significantly higher volume density of eosinophils than did RWs receiving inhaled steroids (CW median, 2.72% [range, 0.12–3.52%] vs. RW median, 0% [range, 0–1.99%]; $P < 0.05$). Moreover, CWs receiving inhaled steroids had a higher volume density of tissue eosinophils compared with CWs not receiving inhaled steroids, although this was not statistically significant (median for those receiving inhaled steroids, 2.72% [range, 0.12–3.52%] vs. those not receiving inhaled steroids, 0.35% [range, 0–1.12%]; $P = 0.09$) (Figure 4c).

Relationship between RBM Thickness and Eosinophilic Inflammation

When all subjects were considered together, there was a significant, positive relationship between RBM thickness and eosinophilic inflammation (Spearman $r = 0.34$, $P < 0.05$). When only CWs were considered, there was a similar trend (Spearman $r = 0.48$, $P = 0.07$) (Figure 5). There was no relationship between RBM thickness and eosinophilic inflammation when RWs or control subjects were considered separately.

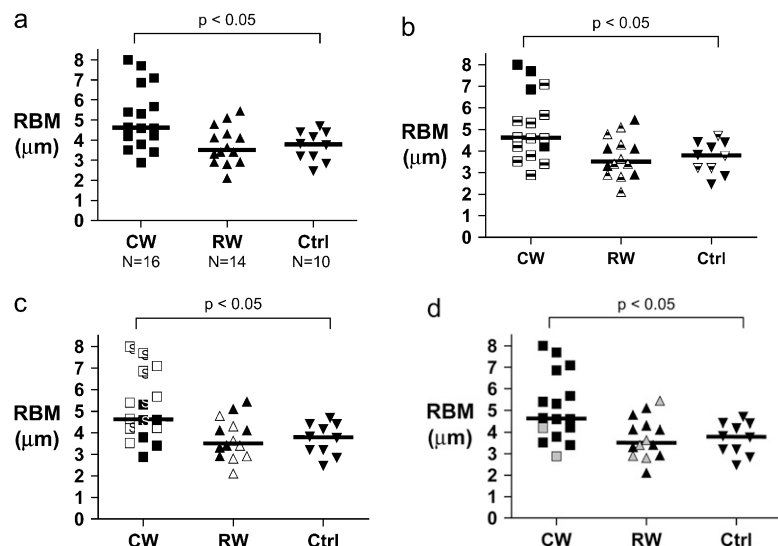


Figure 1. Increased thickness of the reticular basement membrane (RBM) in confirmed wheezers (CW), compared with reported wheezers (RW) and control subjects (Ctrl). *Large horizontal bars* denote medians for all patients throughout. (a) *Solid symbols* show values for all patients; (b) *open symbols with small bar* identify atopic patients; (c) *open symbols* are patients taking corticosteroids either orally (S) or by inhalation (unmarked); (d) *gray-shaded symbols* represent viral wheezers.

DISCUSSION

The present study is the first to suggest a time period of appearance of increased RBM thickness and eosinophilic inflammation, specifically in preschool wheezers, at a median age of 29 months. We did not find a relationship between atopic status and RBM thickness or eosinophilic inflammation. However, there was an early positive relationship between RBM thickness and tissue eosinophilia. Although our group of preschool CWs had a significantly thicker RBM than nonwheezing control subjects, it was not as thick as that which we reported in older children with a median age of 10 years with established asthma (5) (*see* Figure 3).

Increased RBM Thickness in Preschool CWs

Cohort studies that have reported lung function abnormalities in wheezers have either shown initial abnormal lung function at 3 years in persistent wheezers (10) or early abnormalities in infancy (19) that are not maximal until school age (9, 19). Similarly, we have previously shown the absence of RBM thickening in wheezy infants, despite the presence of symptoms and reversible airflow obstruction (7). Here, we demonstrate that by 3 years of age there is evidence of increased RBM thickening. We acknowledge the overlap in the age range of the patients in the current study (5 mo to 5 yr) with that of

our previous report of infant wheezers (3 mo to 2 yr). It was necessary to have some overlap to maximize numbers in each group, especially of control subjects. However, when subjects from the current study were subdivided according to age using 24 months as a cutoff, a strong trend remained for the RBM of CWs older than 24 months to be thicker than their age-matched controls. The loss of statistical power was due mainly to only five control subjects remaining in this age group. In agreement with our earlier findings in infants (7), RBM thickness was similar in CWs and control subjects younger than 24 months. Because it has also been shown in a previous study that RBM thickening becomes maximal by school age (5), it would be reasonable to speculate that the period between 1 and 3 years may be the most appropriate time to intervene in a strategy aimed at minimizing both the pathologic (5) and lung function abnormalities that are expressed clearly by the age of 6 years (11), and which subsequently persist (9). Although actual values of RBM thickness in the current preschool and previous infant study (7) appear to be similar for wheezers and control subjects, the values cannot be compared directly because the biopsies in each study were taken from different airway generations, which we can demonstrate have distinct RBM thicknesses (unpublished data). Moreover, the tissues in the two studies were processed, cut, and stained using completely different techniques, one for electron microscopy and the other for light microscopy.

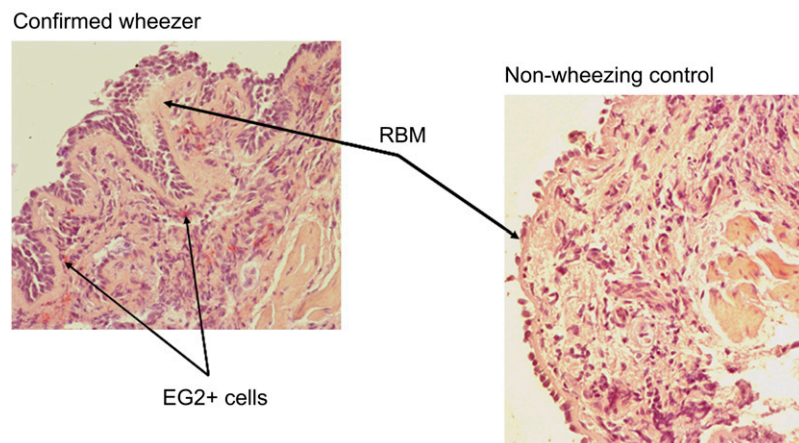


Figure 2. Increased reticular basement membrane (RBM) thickness and EG2⁺ cells in an endobronchial biopsy from a confirmed wheezer compared with a nonwheezing control subject.

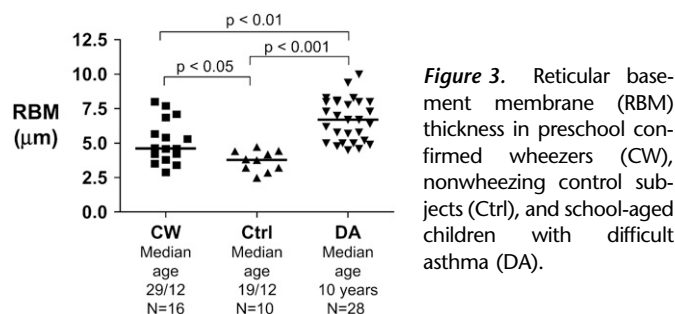


Figure 3. Reticular basement membrane (RBM) thickness in preschool confirmed wheezers (CW), nonwheezing control subjects (Ctrl), and school-aged children with difficult asthma (DA).

Increased Eosinophilic Inflammation in Preschool CWs

It is clear that inhaled steroids are not disease modifying in this age group, either when given intermittently (20) or continuously (21, 22). In our study, which included only patients with severe symptoms, subjects taking inhaled steroids also had the thickest RBM and highest eosinophil counts (Figures 1b and 3b). The high eosinophil counts may be the result of poor adherence to therapy, but they also may be because of poor drug delivery in this age group, failure to use a sufficient steroid dose, or, perhaps, reduced efficacy of steroids. It may also be that the patients selected to receive inhaled steroids were the most likely to be persistent wheezers progressing to confirmed asthma. RBM thickness has only been shown to decrease after prolonged steroid therapy in adults (23). Its increased thickness in our CWs is therefore likely a true reflection of disease at this early stage. Stopping steroid therapy before bronchoscopy was not justified in

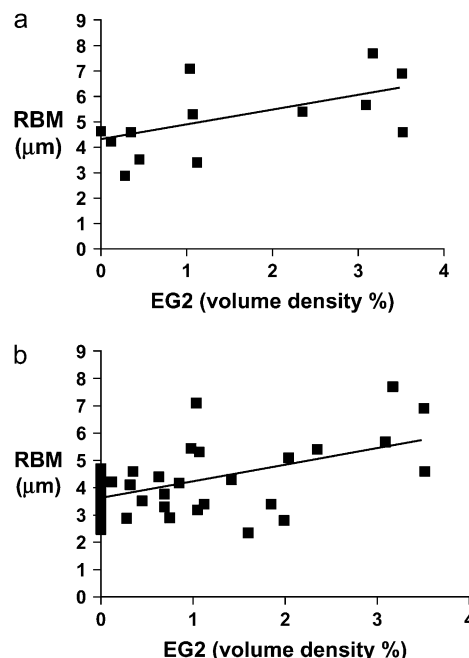


Figure 5. Positive relationship between reticular basement membrane (RBM) thickness and EG2⁺ cells. (a) Confirmed wheezers ($r = 0.48$, $P = 0.07$); (b) confirmed wheezers, reported wheezers, and control subjects ($r = 0.34$, $P < 0.05$).

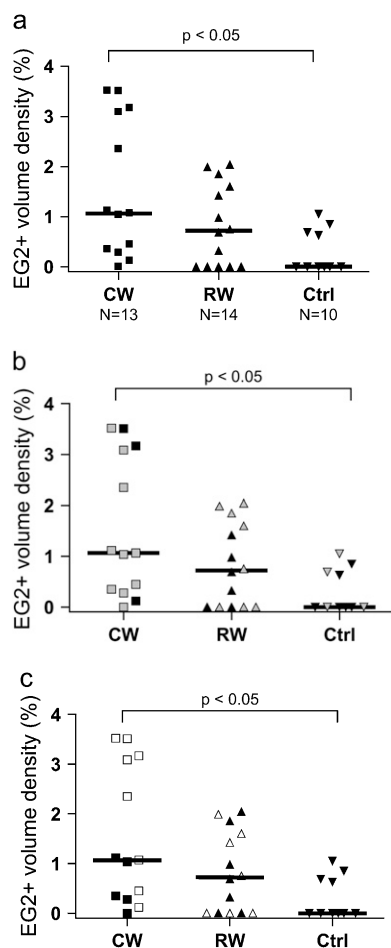


Figure 4. Increased volume density of EG2⁺ cells in confirmed wheezers (CW) compared with reported wheezers (RW) and control subjects (Ctrl). (a) All patients; (b) atopic patients shown by gray-shaded squares; (c) open squares represent patients taking inhaled steroids.

view of symptom severity, but we do not consider that this would have altered the overall message: if anything, we would have underestimated the extent of eosinophilia.

Positive Relationship between Eosinophilic Inflammation and RBM Thickness

In animal studies, eosinophilic inflammation is a requirement for the development of abnormal collagen deposition (24). In humans, tissue eosinophilia and RBM thickening may be present concurrently in eosinophilic conditions other than asthma (e.g., eosinophilic bronchitis) (25), and in adults with asthma an association between eosinophilia and RBM thickening has been demonstrated (26). Although the functional significance of this relationship in relation to preschool wheeze and asthma is unclear, the direct positive relationship that we demonstrate between eosinophilic inflammation and RBM thickness is the first to be reported in children. Finally, in contrast to the findings of Barbato and colleagues, we did not find an association between atopy and RBM thickening or eosinophilic inflammation (2). The presence of increased eosinophilic inflammation in preschool CWs, but not in infants with a median age of 12 months (7), could be attributable to differences in the “maturity” of the immune system at these times. The size and nature of an inflammatory response mounted in response to allergen exposure in infancy are likely different compared with that seen in the older child with a fully developed immune response (27). The airway in the individual who may develop asthma is likely normal at birth, but likely abnormal by the time the child reaches school age. Although the mechanisms driving this change are unknown, they may include the following: (1) developmental changes in the immune system, leading to altered immune responses to allergens (27); (2) increased aeroallergen exposure with the passage of time, leading to sensitization in the first 3 years of life, which is associated with persistent symptoms (28); (3) the effects of repeated viral infections leading to altered immune responses; and (4) the effects of

pollutants, including environmental tobacco smoke, on the immature airway. The elucidation of the specific mechanism(s) is critical to the development of treatment aimed at averting the progression from intermittent to continuous symptoms.

Limitations of the Study

Lack of lung function data. Lung function measurements in infants and preschool children are difficult to perform, and although there were established standards for infant lung function, there were none for preschoolers at the time of commencement of the present study, and we did not determine it (29, 30). We acknowledge the lack of preschool lung function data as a weakness of the study. However, to minimize the effect of an absence of lung function measurements, we tried to clinically characterize the patients fully and, for this purpose, included the use of a video questionnaire to confirm wheeze. Furthermore, in routine clinical practice, characterization of preschool wheeze has to be performed without recourse to lung function.

Control subjects. Only two of our control subjects were asymptomatic; the remainder had upper airway symptoms for which they were undergoing a clinical bronchoscopy. In the latter, there were no lower airway symptoms, specifically wheeze. Ethically, it was impossible to obtain biopsies from healthy, asymptomatic preschool children. Of note, all previous pediatric biopsy studies have included children with lower airway symptoms as controls (2, 3, 5, 6, 15, 31). Ours is the first to include only those with upper airway symptoms.

Uncertain diagnosis. All subjects underwent a clinically indicated bronchoscopy as a result of our previous demonstration of the benefits of bronchoscopy in preschool children with severe wheeze (13). However, EB was performed as a research procedure. At the time of biopsy, we could not know which children would go on to develop asthma and which would outgrow their symptoms. However, to elicit as accurately as possible a time when the pathologic features of asthma appear, the patients were intentionally selected to include those that were clinically deemed most likely to develop asthma in school age (32, 33). It was not possible to include patients with milder symptoms, because bronchoscopy would not have been indicated clinically. Moreover, longitudinal studies that have followed children with asthma to adulthood have shown tracking of abnormal lung function, specifically in those with severe symptoms in childhood (34, 35). Although clinically all subjects recruited were those with severe, recurrent wheeze, to increase our confidence in identifying a group most likely to develop future asthma, an objective confirmation of wheeze was sought by using the video questionnaire. This did not exclude RWs as later developing asthma but allowed greater certainty in identifying a group, the CWs, as likely developing asthma in the future. The overlap in results between CWs and RWs, and RWs and control subjects, suggests that the RWs probably included a less "clean" group of preschool children, some of whom may not have had true wheeze. A similar "in between" group has also been reported previously in relation to lung function, whereby no difference in airway resistance was found between preschool children with parent-reported, but unconfirmed, wheeze and those that had never wheezed. However, those with confirmed wheeze had increased airway resistance (36). The presence of a thicker RBM in subjects with interval, not viral, wheeze, when compared with control subjects, highlights the importance of symptom pattern in determining likely future asthma. This is in accordance with findings from cohort studies (37). We propose to follow up all these children into school age, and rephenotype them retrospectively to determine the clinical utility of these pathologic markers in predicting future asthma.

Other features of airway remodeling. Smooth muscle was not assessed because its quantification in school-aged children with

established asthma has not yet been reported and also because only 15 of the 40 patients recruited had smooth muscle present in their biopsy. Epithelial integrity could not be assessed because intact epithelium of 1 mm in length was rarely present. It remains unclear whether this was a disease effect or a result of taking biopsy samples with smaller forceps than those used in older children. Additional markers of inflammation were not investigated because the biopsies obtained using 1-mm forceps were small and limited the number of tissue sections. The median area of the good-quality biopsies was 0.23 mm², the range was 0.025–1.28 mm². Analysis of the tissue remaining demonstrated that there were insufficient patients left per group to allow meaningful statistical analysis. Individual components of the RBM, such as tenascin, which in adult studies have been shown to be increased in asthma (38), could not be investigated in these biopsy samples from preschool children because alternative techniques of fixing and processing are required. However, we have previously shown that the ultrastructural composition of the RBM is similar in adults, children, and infants with asthma and control subjects (39).

In summary, we have demonstrated that two characteristic pathologic features of asthma, thickening of the RBM and eosinophilic inflammation, are present in a group of preschool children, median age 29 months, with a history of confirmed, severe, recurrent wheeze. This also appears to relate to the time when lung function abnormalities appear in preschool persistent wheezers. Both findings indicate that this is a critical time period when an intervention could potentially attenuate such pathology and impact on the subsequent functional and clinical expression of asthma.

Conflict of Interest Statement: S.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.N.P. received £500 in consultancy fees from AstraZeneca in 2005, £300 from AstraZeneca in 2005, and \$1,000 from Kyorin Pharmaceutical, as well as return air travel to Japan and hotel accommodations for 4 nights from Kyorin Pharmaceutical in 2006. J.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Z.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.G.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.K.J. received \$3,000 a year over the last 3 years in advisory board fees from GlaxoSmithKline (GSK), \$40,000 over the last 3 years in lecture fees from GSK, and \$750,000 from GSK, \$120,000 from Merck, and \$140,000 from AstraZeneca in grants, and invested \$20,000 in buying shares of GSK. P.K.J.'s institution received grants from Pfizer, Inc., Mitsubishi Pharma Corp., GSK UK, Novartis, Horsham, Boehringer Ingelheim, AstraZeneca, Microbia, Inc., Merck, Sharp, and Dohme, Pharmaxis Ltd, Chiron Corporation, Asthmatix, Inc., Intas, Surface Therapeutics Ltd, Alk-Abello A/S, Millennium Pharmaceuticals, Inc., Oxagen Ltd, Sirtis Pharmaceuticals, Inc., Sanofi Pasteur, Centocor, Inc., Medimmune, Inc., Domantis Ltd, Tibotec Pharmaceuticals, Bavarian Nordic A/S, Buxco Europe Ltd, Syntaxis Ltd, and Arriva Pharmaceuticals, Inc.

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**Early detection of airway wall remodelling and eosinophilic inflammation
in preschool wheezers**

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Andrew Bush and Peter K Jeffery**

Online data supplement

Methods

Subjects

Children included were aged between 3 months and 5 years and had been referred to the Royal Brompton Hospital with severe, recurrent wheeze and were due to undergo a clinically indicated fibreoptic bronchoscopy (FOB) as part of further investigation of their symptoms.^{E1} All subjects continued on their regular therapy at the time of bronchoscopy. They all had recurrent, severe, noisy breathing, which had been interpreted as wheeze, either when assessed during acute symptoms, or from parental reports. Most had been admitted at least once with acute wheeze, and all had ongoing symptoms despite bronchodilator therapy and previous trials of inhaled or oral steroids. They had had at least 3 severe episodes of wheeze (each episode with symptoms for more than 3 days) in the previous 6 months. The following pattern of symptoms were identified: i) symptoms present on most days, ii) episodic symptoms only with colds, iii) acute exacerbations with colds and interval symptoms between colds. None of the children were included in our previous study of infant asthma.^{E2}

Although previous hospital admission with wheeze was not an inclusion criterion, the majority of subjects had been admitted at least once with acute wheeze. All patients had persistent symptoms despite a previous trial of bronchodilator therapy. They had also all had a previous trial of inhaled or oral steroid therapy. The minimum duration of the trial of inhaled steroids was 2 months. The dose of inhaled steroids used during the trial of therapy was variable and parents were not always certain of the dose used. All patients

continued to have symptoms in one of the three patterns identified above, despite previous trials of therapy, or their currently prescribed therapy.

In order to ensure those included were not more likely to have a diagnosis other than asthma as the main explanation for their wheeze, the following exclusion criteria were applied: i) subjects with isolated cough without associated noisy breathing, ii) subjects whose main problem was recurrent lower respiratory tract infection, iii) those on long term oxygen therapy, iv) those with chronic lung disease of prematurity.

Although details concerning current treatment, including inhaled and oral steroid therapy were obtained, all subjects continued on their regular therapy, including inhaled and/or oral steroids at the time of bronchoscopy.

Controls

Controls were obtained in two ways. Firstly, (the majority) controls were patients who were undergoing a clinically indicated bronchoscopy for further assessment of troublesome stridor or to assess the degree of airway compression from a vascular ring. Parental informed consent was obtained to perform research biopsies in these cases. Secondly, two controls were recruited from patients undergoing a right sided diagnostic cardiac angiogram. Parents were asked for consent to perform a research bronchoscopy and endobronchial biopsies before the cardiac angiogram. These two patients did not have any respiratory symptoms. ^{E3} None had a history of wheezing or lower respiratory symptoms.

Informed consent

The study was approved by the Royal Brompton and Harefield NHS Trust ethics committee, and fully informed parental consent was obtained to take research biopsies, prior to recruitment.

Video Questionnaire

All parents (of both subjects and controls) were shown a previously validated video questionnaire (VQ) ^{E4} in order to obtain objective confirmation of the presence of wheeze. It contained 4 clips of infants and children demonstrating examples of wheeze (male, aged 6 years), stridor (male, aged 6 months) and 2 different upper respiratory noises (both females aged 10 and 24 months). The subjects and controls were divided into 3 groups according to findings from the VQ:

1. Confirmed wheeze – subjects whose parents had reported wheeze as their child's main symptom and identified wheeze from the VQ.
2. Reported wheeze – subjects whose parents reported wheeze as their child's main symptom, but after being shown the VQ, did not identify wheeze from it.
3. Controls – subjects whose parents reported stridor or no respiratory symptoms and did not identify wheeze from the VQ.

School aged difficult asthmatics

RBM thickness in EB from 29 school-aged children (median age 10 [range 6-16] years) with established difficult asthma that had been collected for a previous study ^{E5} was measured again for this study to compare RBM thickness in preschool wheezers and controls to older children with established asthma.

Atopic status

Atopy was defined as the presence of eczema in the child and / or at least one positive IgE RAST to the following food or aero-allergens: milk, egg, peanut, house dust mite, cat, dog and grass pollens.

Fibreoptic bronchoscopy and endobronchial biopsy

All children, wheezers and controls, were due to undergo a clinically indicated bronchoscopy. However, not all subjects were due to undergo clinically indicated EB. This was performed as an additional research procedure. Fully informed consent was obtained from the parents of all subjects to take endobronchial biopsies specifically for this study. FOB and EB were performed under general anaesthesia as previously described.^{E6} A size 2.8mm or 3.6mm bronchoscope (Olympus, KeyMed, UK) was used. Both have the same sized instrument channel, allowing size 1mm rat-tooth forceps (Olympus, Key Med, UK, serial number FB-56D-1) to be used. Upto 4 EB were taken from the third or fourth generation sub-segmental bronchi of the right lower lobe.

Biopsy processing

Up to 4 EB were immersed in 10% formal saline and processed to paraffin blocks between 4 and 24 hours after fixation. Three 5µm thick sections were taken at 25-50µm step intervals, dependent on biopsy size and were stained using haematoxylin and eosin in order to assess morphology, overall biopsy quality and to quantify the RBM. Inflammatory cell phenotypes were identified by immunostaining for leukocytes (CD45+), neutrophils (neutrophil elastase) and eosinophils (EG2).^{E7}

Immunostaining of inflammatory cells

Identification of inflammatory cells including CD45+ cells, neutrophils (using neutrophil elastase) and eosinophils (using EG2) within paraffin embedded endobronchial biopsies was performed using the EnVision-alkaline phosphatase (EV-AP) technique (DAKO Ltd, Cambridge, UK), and identification of CD4+ and CD8+ cells was performed using the EnVision Peroxidase staining method (DAKO Ltd, Cambridge, UK), according to the supplier's instructions, and as previously described.^{E7}

Quantification of RBM thickness

All sections were coded and measured in random order by a single observer (SS), unaware of their origin. Biopsies of good morphological quality (those that included recognisable epithelium, at least 1mm in length of RBM, and submucosa) were chosen for analysis from haematoxylin and eosin sections by SS. These were then given to JZ who allocated a number to each in a random fashion and cut new sections from the respective blocks for immunostaining and assigned those random numbers as well. The final RBM and inflammatory cell measurements were performed by SS in these coded sections using computer-aided image analysis (NIH image 1.55; National Institute of Health, Bethesda, MD). At least 40 measurements of RBM thickness were made 20µm apart, using a previously validated method.^{E8} The geometric mean of all measurements was used to represent the RBM thickness of that section.

Quantification of subepithelial inflammatory cells

Sections were coded and counted in random order by a single observer (SS), unaware of their origin. Areas of the section containing a given density of immunostained cells were assessed by the validated technique of point counting^{E9} using light microscopy, as previously described.^{E5} Briefly, at a magnification of x400, and using an eye-piece graticule containing 100 equidistant points (Pyser-SGI, Kent, UK), all evaluable areas of submucosa below the epithelium, excluding areas of smooth muscle, vessels and glands, were analysed. Points overlying positively staining cells were counted as positive and those overlying other areas as negative. For all cell types, one section from each biopsy was counted for each subject. Sections were considered suitable for analysis if the components of epithelium, RBM and submucosa could be easily identified. At least 200 points were counted per subject.^{E9} The volume density of the cell of interest was calculated as the sum of all positive points divided by the total number of points, multiplied by 100, the result was expressed as a percentage.^{E9}

Variability

The intra-observer variability of measurements was calculated by measuring the same section 4 times on different occasions, at least 3 days apart. This was expressed as the % coefficient of variation (CV). There were no more than 2 biopsies processed in the same way per patient. Therefore between biopsy variability was assessed by examining a section from two biopsies from the same patient, and the level of agreement assessed using a Bland-Altman plot.^{E10} As the tissue was limited and biopsies were relatively small, none had

been cut through at different levels for the purpose of assessment of the within biopsy variability.

Statistical analysis

Non-parametric tests were applied. Comparison between two groups was made using the Mann-Whitney U test. The difference between three or more groups was assessed using the Kruskal-Wallis test, followed by Mann-Whitney U testing if a significant difference was found. A Bonferroni correction was then applied for multiple comparisons. Spearman rank correlation was used to assess associations. A p value <0.05 was considered statistically significant. All data were analysed using the Statistical Package for Social Sciences (SPSS) version 11.5.

Results

RBM thickness in children older than 24 months

In order to distinguish infant and preschool wheezers maximally, subjects were sub-divided according to age using 24 months as a cut-off. The RBM was thicker in CW older than 24 months compared to age-matched controls (median RBM thickness CW 5.0 [range 3.4-8.0] μm vs Ctrl 3.76 [3.2-5.5] μm , $p=0.058$) (figure E1). There was no difference in RBM thickness between CW and Ctrl less than 24 months.

RBM thickness and atopy

When all wheezers and controls were divided according to atopic status, RBM thickness was similar in atopic and non-atopic children (atopic median 3.6 [2.0-5.7] vs non-atopic median 3.8 [2.5-8.0] μm , NS), figure E2. There was no difference in RBM thickness between atopic and non-atopic CW, and atopic and non-atopic controls (figure E3).

Subepithelial inflammation and atopy

When all wheezers and controls were considered together, there was no difference in CD45+, EG2+, NE+, CD4+ or CD8+ between atopic and non-atopic patients (figure E4). There was also no difference in eosinophilic inflammation between atopic wheezers and non-atopic wheezers, and no difference between atopic and non-atopic controls (figure E5).

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Figure E1. Trend towards a thicker reticular basement membrane in confirmed wheezers (CW) older than 24 months compared to age-matched controls (Ctrl)

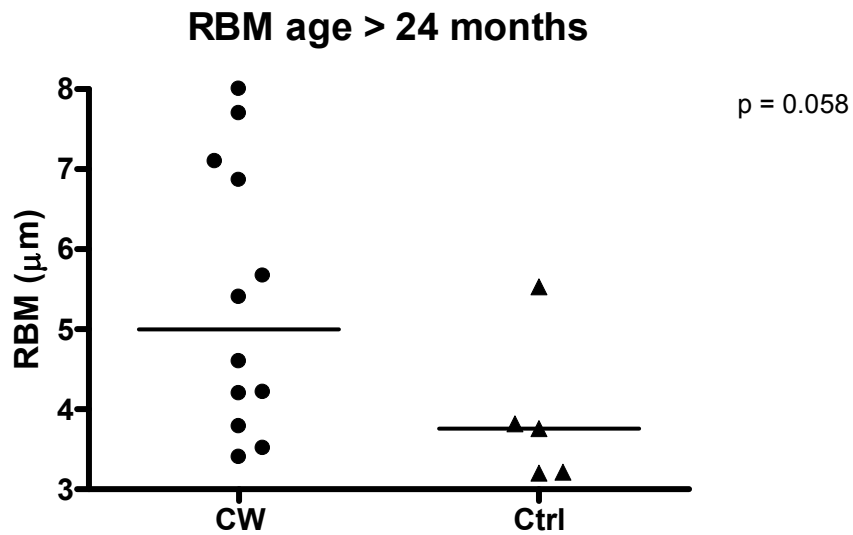


Figure E2. Similar RBM thickness in atopic and non-atopic patients

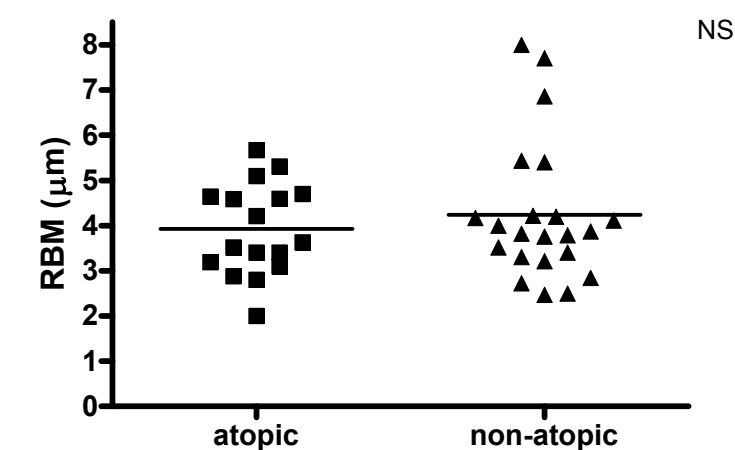
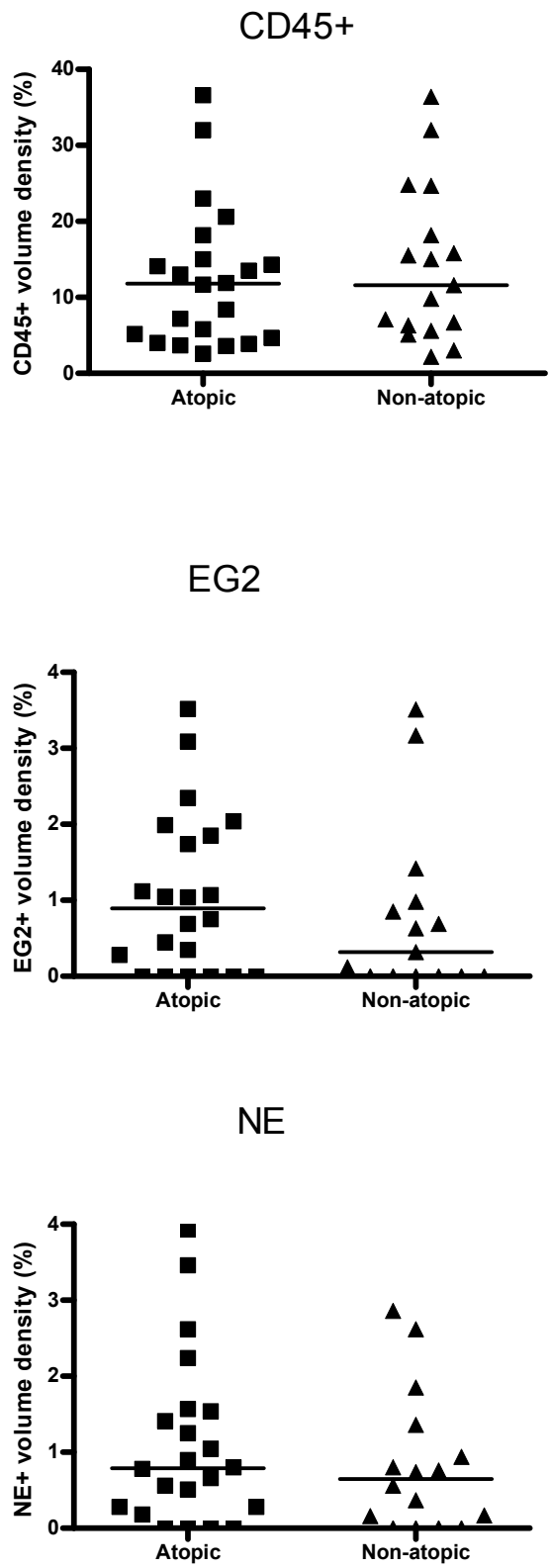
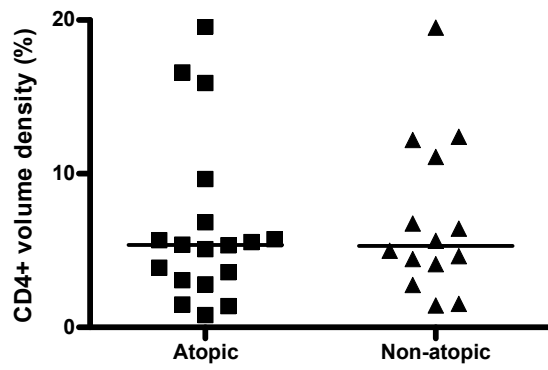


Figure E4. Similar volume density of inflammatory cells (CD45, EG2, NE, CD4 and CD8) in atopic compared to non-atopic preschool children



CD4



CD8

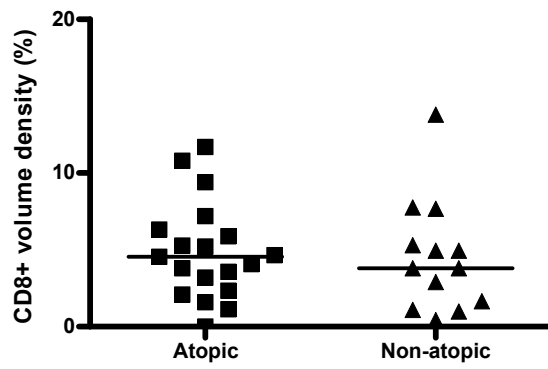


Figure E5. Similar volume density of EG2+ cells in a) atopic wheezers and non-atopic wheezers and b) atopic and non-atopic controls

