# Increased airway smooth muscle in preschool wheezers who have asthma at school age

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**Background: Increased airway smooth muscle (ASM) is a feature of established asthma in schoolchildren, but nothing is known about ASM in preschool wheezers.**
**Objective: We sought to determine endobronchial biopsy specimen ASM area fraction in preschool wheezers and its association with asthma at school age.**
**Methods: ASM area, reticular basement membrane thickness, and mucosal eosinophil and ASM mast cell values were quantified in endobronchial biopsy specimens previously obtained from preschool children undergoing clinically indicated bronchoscopy: severe recurrent wheezers (n = 47; median age, 26 months) and nonwheezing control subjects (n = 21; median age, 15 months). Children were followed up, and asthma status was established at age 6 to 11 years. Preschool airway pathology was examined in relation to asthma at school age.**
**Results: Forty-two (62%) of 68 children had 1 or more evaluable biopsy specimens for ASM. At school age, 51 of 68 children were followed up, and 15 (40%) of 37 preschool wheezers had asthma. Children who had asthma and an evaluable biopsy specimen had increased preschool ASM area fraction (n = 8; median age, 8.2 years [range, 6-10.4 years]; median ASM, 0.12 [range, 0.08-0.16]) compared with that seen in children without asthma (n = 24; median age, 7.3 years [range, 5.9-11 years]; median ASM, 0.07 [range, 0.02-0.23]; *P* = .007). However, preschool reticular basement membrane thickness and mucosal eosinophil or ASM mast cell values were not different between those who did or did not have asthma at school age.**
**Conclusion: Increased preschool ASM is associated with those children who have asthma at school age. Thus a focus on early changes in ASM might be important in understanding the subsequent development of childhood asthma. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)**

***Key words:*** *Preschool wheeze, airway smooth muscle, asthma, pediatric, pathology*

Airway wall inflammation and structural changes (ie, remodeling), which include increased reticular basement membrane (RBM) thickness and increased airway smooth muscle (ASM), are pathologic characteristics of asthma.[1] In particular, alterations in ASM are most consistently associated with abnormal lung function. ASM hypertrophy and hyperplasia in school-aged and adult asthmatic patients have been significantly associated with bronchodilator responsiveness[2-4] and airway hyperresponsiveness.[5] Furthermore, the proportion of ASM increases as airflow limitation worsens.[6,7] ASM mast cell infiltration distinguishes asthma from eosinophilic bronchitis in adults and correlates with airway hyperresponsiveness.[8-10]

Although one third of all preschool children wheeze,[11] only half of these will have persistent symptoms and be given a diagnosis of asthma at school age.[12] However, our understanding of airway pathology in preschool children and its relationship to future asthma remains limited. Increased RBM thickness and eosinophilic inflammation have been demonstrated in endobronchial biopsy (EB) specimens from preschool children with severe recurrent wheeze aged 2 to 3 years when compared with that seen in preschool control subjects with no lower respiratory tract symptoms.[13] Although increased ASM mass is a feature of established asthma in adults and children as young as 7 years,[1,3] nothing is known about ASM in preschool wheezers or the long-term significance of airway remodeling in preschool wheezers.

We have previously performed bronchoscopy and obtained EB specimens in wheezy preschool children attending our clinic, but at that time, their future clinical outcome was not known.[13] We hypothesized that children with severe recurrent wheeze at preschool age would exhibit increased ASM compared with that seen in nonwheezing control subjects and that preschool airway pathology would be predictive of the later development of asthma at school age.

We therefore followed up a prospectively recruited group of preschool wheezers and control subjects who underwent a clinically indicated bronchoscopy and EB between 2002 and 2005 to school age (2009-2010) to establish the presence or absence of asthma and then examined the relationships between preschool airway pathology and asthma status at school age.[13]

Some of the results in these studies have been reported in the form of abstracts.[14,15]

## METHODS

### Preschool subjects

Full subject details have been reported previously and are fully described in the Methods section in this article's Online Repository at www.jacionline.org.[13] EB specimens were obtained from preschool children undergoing

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*Abbreviations used*
ASM: Airway smooth muscle
EB: Endobronchial biopsy
$FENO_{50}$: Fraction of exhaled nitric oxide at 50 mL/s
FRC: Functional residual capacity
RBM: Reticular basement membrane
ROC: Receiver operating curve
$S_{cond}$: Conducting airways inhomogeneity
SPT: Skin prick test

clinically indicated bronchoscopy between 2002 and 2005: severe recurrent wheezers (n = 47; median age, 26 months [range, 6-58 months]) and nonwheezing control subjects (n = 21; median age, 15 months [range, 3-42 months]). We measured total IgE levels and RAST results to 3 food allergens and 5 aeroallergens. Children were further divided into confirmed or reported wheezers based on parental identification of wheeze on a video questionnaire.[16] The majority of nonwheezing control subjects had bronchoscopy for assessment of upper airway problems.

## Follow-up at school age

**Clinical diagnosis of asthma.** Children were followed up at age 6 to 11 years (Fig 1). Caregivers completed International Study of Asthma and Allergy in Children core questionnaires for asthma, eczema, and allergic rhinitis.[17] Asthma was defined as both a positive caregiver response to the question "Has your child had wheezing or whistling in the chest in the last 12 months?" and a caregiver report of doctor-diagnosed asthma ever.[18-23] Caregivers were asked to identify wheeze at school age using a validated video questionnaire.[24] Two asthma clinical prediction indices, the "stringent index" derived from the Tucson cohort and the wheeze severity score, were calculated from data obtained at preschool recruitment.[25,26] For the stringent index, wheezing apart from colds or peripheral blood eosinophilia were used as the minor criteria. For the wheeze severity score, 3 to 4 episodes of wheeze plus the number of hospital admissions was used to approximate the score.

**Lung function.** Spirometry was performed in accordance with American Thoracic Society guidelines.[27] Multiple-breath washout was measured with a modified Innocor photoacoustic device (Innovision, Odense, Denmark) that has been validated in children older than 5 years.[28,29] Children breathed 0.2% sulphur hexafluoride in air (BOC, Hertford, United Kingdom) until a steady state was reached. The gas supply was then detached during expiration, and patients then breathed room air until the end-tidal sulphur hexafluoride concentration was one fortieth of the starting concentration (0.005%). Washouts were performed 3 times.[30] Washouts were excluded if the measured functional residual capacity (FRC) differed by more than 10% from both of the repeat washouts.[30] Lung clearance index, FRC, acinar airways inhomogeneity, and conducting airways inhomogeneity ($S_{cond}$) values are reported as the mean of 2 or more reproducible measurements.[28]

**Airway inflammation and atopy.** Fraction of exhaled nitric oxide at 50 mL/s ($FENO_{50}$) was measured with a chemiluminescence analyzer (NiOX; Aerocrine AB, Solna, Sweden).[31] Skin prick tests (SPTs) were performed for 3 food allergens (hen's egg, cow's milk, and peanut) and 6 aeroallergens (cat, dog, grass, tree, *Aspergillus fumigatus*, and *Dermatophagoides pteronyssinus*; Soluprick SQ, Alk-Abelló, Hørsholm, Denmark).[32] Positive and negative controls were included.

**Quantification of RBM thickness, ASM, and inflammatory cells.** Full details of bronchoscopy, biopsy processing, immunostaining, and quantification of RBM thickness and subepithelial eosinophil values have been reported previously (and see the Methods section in this article's Online Repository).[13,33] These were quantified again by a different observer (R.O.) using the same techniques for this report. ASM was assessed for the first time in sections stained with hematoxylin and eosin by using 2 techniques: (1) ASM area was represented as a fraction of subepithelial area (LEICA Qwin version 3; see Fig E1 in this article's Online Repository at www.jacionline.org),[34] and (2) the volume fraction of ASM was measured by using point and line intersection counting (see Fig E2 in this article's Online Repository at www.jacionline.org).[3,4] Biopsy specimens of less than 0.2 $mm^2$ were excluded. Each child had the mean recorded ASM from all sections measured. A second blinded observer also quantified the ASM area fraction to assess interobserver repeatability. Mast cells were stained with mast cell tryptase and were expressed as numbers per square millimeter of ASM.[8] ASM was quantified in biopsy specimens from nonasthmatic school-aged children (n = 13) between 5.9 and 14.2 years old to assess the relationship between age and ASM (see the Methods section in this article's Online Repository).

## Statistical analysis

Sample size was opportunistic, and there are no previous data in preschoolers to inform a power calculation. Differences between groups were analyzed by using nonparametric tests. A $P$ value of less than .05 was considered statistically significant. Analyses were performed with MedCalc version 12.1.0 (MedCalc Software, Mariakerke, Belgium) and GraphPad Prism version 5.02 (GraphPad, La Jolla, Calif) software (see the Methods section in this article's Online Repository).

# RESULTS

## Clinical characteristics and asthma status at school age

At school age, 37 of 47 preschool wheezers and 14 of 21 control subjects were followed up. Questionnaires were completed by 51 (75%) of 68 families, and 39 children attended a full research visit (Fig 1). Twelve families would not or could not attend a research visit, but all subsequently agreed to answer a telephone questionnaire. Seventeen children were lost to follow-up. Fifteen (40%) of 37 preschool wheezers and 1 control subject (1/14) had asthma at school age. There was no difference between children who participated in the study and those lost to follow-up in terms of sex ($P$ = .77), age at bronchoscopy and EB ($P$ = .77), or preschool IgE level ($P$ = .99, see Tables E1-E4 in this article's Online Repository at www.jacionline.org). The clinical characteristics of children followed up at school age are detailed in Table I.

## Preschool ASM related to asthma status at school age

Forty children had 1 evaluable biopsy specimen, and 2 children had 2 evaluable biopsy specimens. Reasons for exclusion were no evaluable biopsy specimen (n = 12), biopsy specimen size of less than 0.2 $mm^2$ (n = 7), and no identifiable ASM (n = 7). The intraclass correlation coefficient was 0.78 for the 2 measurement techniques used to determine ASM area fraction[34] and ASM volume fraction.[3,4] The intraobserver repeatability (expressed as the percentage mean coefficient of variation) was better for ASM measured by using computer-aided analysis (7.5%) compared with point and line intersection counting (15%). There was good repeatability between 2 independent observers for ASM area fraction measurements (see Fig E3 in this article's Online Repository at www.jacionline.org).

Of 51 children followed up at school age, 32 had an evaluable EB specimen with ASM visible. Eight of 32 children (7 preschool wheezers and 1 preschool control subject) had asthma at school age, and 24 of 32 (14 preschool wheezers and 10 preschool control subjects) did not. Children with asthma at school age (n = 8) had increased preschool ASM area fraction (Fig 2, *B*, and Table II) and increased ASM volume fraction (median, 0.153 [range, 0.094-0.266] vs median, 0.097 [range, 0.02-0.314]; $P$ = .016).

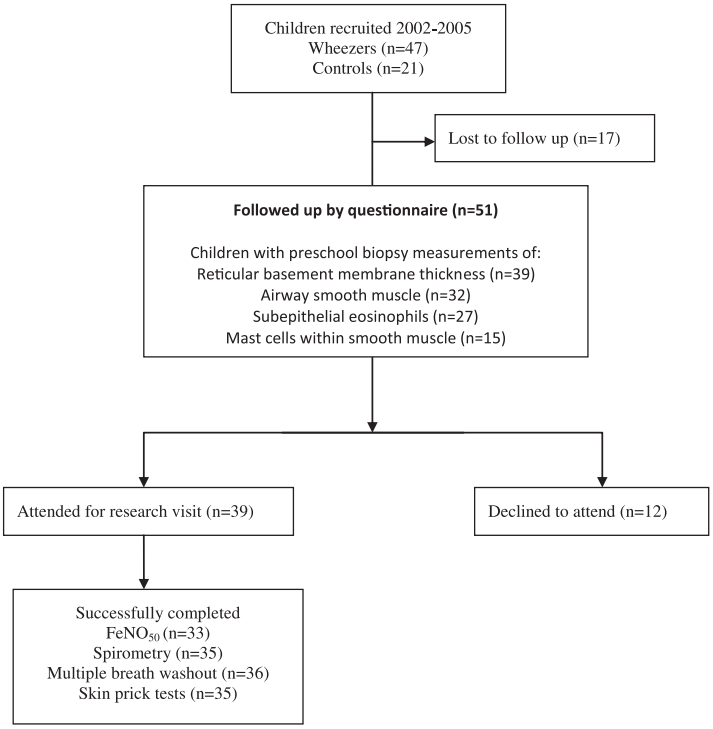


**FIG 1.** Flow diagram showing follow-up at school age (2009-2010) and tests performed in previously recruited preschool children.

The total subepithelial biopsy area measured was larger in those with asthma at school age (median, 0.81 $mm^2$ [range, 0.35-1.27 $mm^2$]) when compared with those without asthma at school age (median, 0.52 $mm^2$ [range, 0.26-1.54 $mm^2$]; $P = .043$). ASM proliferation was assessed based on myocyte proliferating cell nuclear antigen levels in children with remaining biopsy tissue, and no difference was seen between those with (n = 3) and without (n = 7) asthma at school age. However, because the numbers are small, no statistical analysis is feasible (see Fig E4 in this article's Online Repository at www.jacionline.org).

## Age and ASM

Children with asthma were older at the time of EB (28 months) compared with those without asthma at school age (17 months, $P = .01$). To evaluate further the relationship between age and ASM, nonwheezing school-aged children who were undergoing clinically indicated bronchoscopy, mainly for evaluation of upper airway problems, had EB specimens taken for research purposes, and the ASM volume fraction was quantified. There was no correlation between age and ASM volume fraction in nonwheezing preschool children (n = 14) and school-aged children (n = 13) aged 0.25 to 14.2 years (Spearman $r = 0.027$, $P = .89$).

## Relationship between preschool ASM and preschool wheeze

At preschool age, there was no difference in area fraction of ASM between wheezers and nonwheezing control subjects ($P = .97$; Fig 2, *A*) or ASM volume fraction between wheezers and control subjects ($P = .6$). There was also no difference in ASM when preschool wheezers were divided into confirmed (n = 15) or reported (n = 13) wheezers based on parental identification of wheeze on video questionnaires ($P = .16$; see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). Only 5 children with episodic wheeze (ie, wheezing only with colds) had ASM measured at preschool age, and therefore we were unable to assess differences between children with episodic or multiple-trigger wheezing at preschool age (see Fig E5, *B*).

## Preschool RBM thickness and subepithelial eosinophils related to asthma status at school age

Children with severe recurrent wheeze at preschool age had increased RBM thickness ($P = .009$) and subepithelial eosinophil numbers ($P = .01$) when compared with nonwheezing control subjects at preschool age (Fig 2, *C* and *E*). However, preschool RBM thickness ($P = .16$) and subepithelial eosinophil density ($P = .14$) were similar in children who did or did not have asthma by school age (Fig 2, *D* and *F*, and Table II). RBM thickness ($P = .12$) and subepithelial eosinophil numbers ($P = .44$) did not discriminate asthma when the preschool wheezers were considered alone (see Fig E6 in this article's Online Repository at www.jacionline.org).

## Preschool smooth muscle mast cell values and asthma status at school age

Twelve of 32 children did not have smooth muscle in the sections stained with mast cell tryptase. The number of mast cells per smooth muscle area was similar in control subjects and wheezers (Fig 2, *G*). There was no relationship between smooth muscle mast cells and asthma status at school age (Fig 2, *H*, and Table II). There was no correlation between ASM mast cells

**TABLE I.** Clinical characteristics of children followed up at school age

| | Asthma (n = 16) | No asthma (n = 35) | *P* value |
|---|---|---|---|
| Male/female sex | 11/5 | 21/14 | .54 |
| Birth weight (kg) | 3.41 (1.87-4.21) | 3.38 (1.6-4.41) | .57 |
| Gestation (wk) | 39 (30-42) | 40 (34-42) | .51 |
| Age at bronchoscopy (mo) | 28 (3-57) | 17 (3-55) | **.01** |
| Weight at bronchoscopy (kg) | 13.35 (5.6-25) | 11 (5.45-26.4) | **.043** |
| Preschool IgE (IU/mL) | 70 (1-635) | 9 (1-432)* | **.04** |
| Preschool ≥1 positive RAST, no. (%) | 6 (37.5) | 10 (31.3)* | .75 |
| Age at first episode of wheeze (mo) | 14 (1.5-24) | 6.5 (0.5-66) | .21 |
| | | | |
| Wheeze at preschool age | 15 | 22 | |
| Confirmed wheezers | 11 | 10 | |
| Reported wheezers | 4 | 12 | |
| No wheeze | 1 | 13 | |
| Parental history of doctor-diagnosed asthma† | 13 | 17† | **.04** |
| Paternal history of doctor-diagnosed asthma | 5 | 1 | **.01** |
| Maternal history of doctor-diagnosed asthma | 5 | 7 | .28 |
| | | | |
| Age at follow-up (y) | 8.2 (6-10.4) | 7.3 (5.9-11) | .1 |
| Reported wheeze in last 12 mo | 16 | 7 | |
| Wheeze pattern | | | |
| Episodic (with colds) | 3 | 5 | |
| Multiple trigger | 13 | 2 | |
| Frequency of wheeze | | | |
| None | 0 | 28 | |
| 1-3 attacks of wheezing | 4 | 4 | |
| 4-12 attacks of wheezing | 8 | 2 | |
| >12 attacks of wheezing | 4 | 1 | |
| Exercise-induced wheeze | 13 | 2 | |
| Dry cough at night‡ | 14 | 7 | |
| Wheeze assessed by VQ at school age | 12 | 27 | |
| Identified wheeze at school age | 9 (75%) | 8 (30%) | |
| | | | |
| No. of hospital admissions with wheeze | 17 (0-160) | 3 (0-20) | **.005** |
| | | | |
| Use of ICS | 13 | 4 | |
| Dose of ICS (μg beclomethasone)§ | 400 (200-1200)§ | 300 (200-400) | |
| | | | |
| Eczema | 12 | 4 | **.0001** |
| Allergic rhinitis | 7 | 9 | **.001** |

*ICS*, Inhaled corticosteroid; *V/Q*, video questionnaire.
Data are reported as the median and range. The Mann-Whitney *U* test was used for ordinal data, and the Fisher exact test was used for categorical data. Boldface represents statistically significant results.
*Results not available for 3 children.
†Family history was unknown in 2 children because both were adopted at birth (previously in the control group).
‡Dry cough at night apart from a cough associated with a cold or chest infection.
§Or beclomethasone equivalent (2 parents did not know the ICS dose).

and either ASM area or volume fraction (see Table E5 in this article's Online Repository at www.jacionline.org).

## Using ASM to predict asthma at school age in preschool wheezers

When preschool wheezers were considered alone, those in whom asthma developed had increased ASM area fraction (n = 7; median, 0.127 [range, 0.083-0.156]) compared with those in whom asthma did not develop (n = 14; median, 0.058 [range, 0.038-0.163]; $P = .006$; see Fig E6 in this article's Online Repository at www.jacionline.org). The positive predictive value of ASM area fraction of greater than 0.1 at preschool age for asthma at school age was 75% (35% to 97%), and the negative predictive value was 92% (64% to 100%, Table III).[25,26] The area under the receiver operating curve (ROC) was 0.88 (95% CI, 0.66-0.98; $P < .0001$; Fig 3). ASM volume fraction showed similar results, (see Fig E7 in this article's Online Repository at www.jacionline.org).

## Exhaled nitric oxide levels, atopy, and lung function at school age

Of children who attended a research visit at school age, those with asthma at school age had higher $FENO_{50}$ values ($P = .02$) and were more likely to have 1 or more positive SPT responses ($P = .002$) when compared with those without asthma (Table IV). There was no difference in spirometric, lung clearance index, or FRC values between children with and without asthma at school age (Table IV). However, children with asthma did have increased $S_{cond}$ values, a marker of ventilation inhomogeneity in the conducting airways (n = 7; 0.043 [range, 0.003-0.06]), when compared with those without asthma (n = 22; 0.014

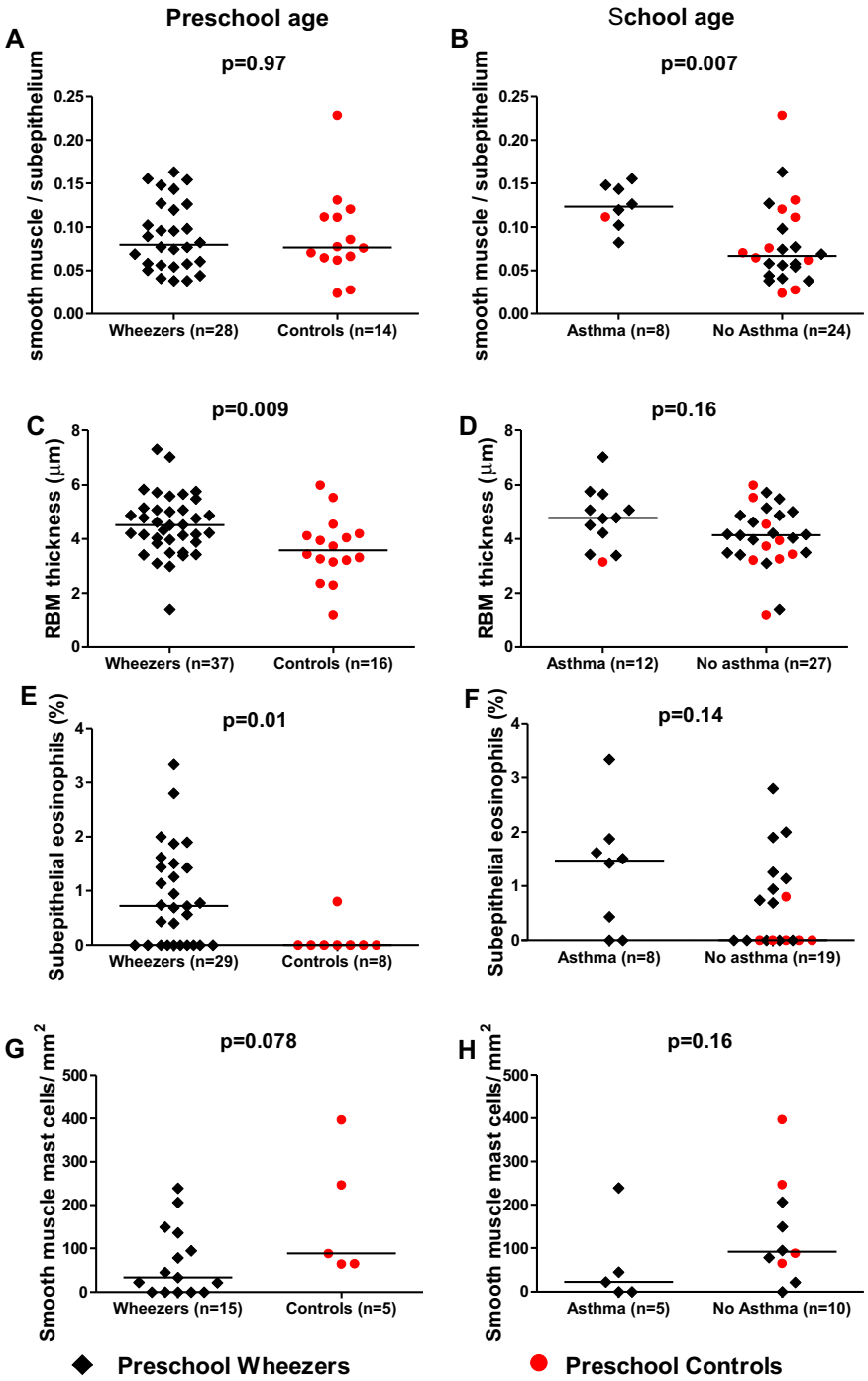


**FIG 2.** Preschool airway pathology and its relationship to preschool wheeze and asthma at school age. **A**, No difference in ASM area fraction between preschool wheezers and control subjects. **B**, Increased preschool ASM area fraction in school-aged asthmatic subjects. **C**, Increased RBM thickness in preschool wheezers. **D**, No difference in preschool RBM thickness in school-aged asthmatic subjects. **E**, Increased subepithelial eosinophil numbers at preschool age. **F**, No difference in preschool subepithelial eosinophil numbers in school-aged asthmatic subjects. **G**, No difference in ASM mast cell numbers between preschool wheezers and control subjects. **H**, No difference in ASM mast cell numbers in school-aged asthmatic subjects. Analysis was performed with the Mann-Whitney *U* test.

[range, −0.027 to 0.03]; $P = .049$; Table IV). $\text{FENO}_{50}$ values at school age correlated with preschool ASM area fraction (Spearman $r = 0.58$, $P = .01$) but showed no relationship with RBM thickness in preschool wheezers (Fig 4).

## DISCUSSION

We report for the first time that ASM, expressed either as area or volume fraction, was discriminatory for those preschool wheezers who subsequently had asthma by school age. In

**TABLE II.** Preschool airway pathology related to the presence or absence of asthma at school age

| | Asthma | No asthma | *P* value |
|---|---|---|---|
| ASM area/subepithelial area | n = 8, 0.12 (0.08-0.16) | n = 24, 0.066 (0.024-0.23) | **.007** |
| V/v − ASM/subepithelium | n = 8, 0.153 (0.094-0.266) | n = 24, 0.097 (0.02-0.314) | **.016** |
| V/S $\mu m^2/\mu m^3$ | n = 8, 73.45 (28.3-127.7) | n = 24, 34 (9.3-374) | .15 |
| RBM thickness (μm) | n = 12, 4.77 (3.14-7.02) | n = 27, 4.14 (1.21-5.99) | .16 |
| Subepithelial eosinophil volume density (%) | n = 8, 1.47 (0-3.33) | n = 19, 0 (0-2.8) | .14 |
| ASM mast cells (no./$mm^2$) | n = 5, 22.9 (0-239.2) | n = 10, 91.8 (0-396.6) | .16 |

*V/S*, Volume fraction of smooth muscle indexed to surface area of RBM; *V/v*, volume fraction of smooth muscle/subepithelium.
Data are reported as the number of children with available data *(n)*, median, and range. The Mann-Whitney *U* test was used. Boldface represents statistically significant results.

**TABLE III.** Sensitivity, specificity, positive and negative predictive values, and likelihood ratios for a proportion of ASM area greater than 10% in the EB specimen compared with the clinical predictive stringent index and wheeze severity score in preschool wheezers

| | Sensitivity (%) | Specificity (%) | NPV (%) | PPV (%) | *P* value | Likelihood ratio |
|---|---|---|---|---|---|---|
| ASM area >10%* | 86 (42.1-99.6) | 85.7 (57.1-98.2) | 92.3 (63.9-99.8) | 75 (34.9-96.8) | .003 | 6 |
| Stringent index[25] | 78.8 (49.2-95.4) | 45 (23.1-68.5) | 50 (28.2-71.8) | 75 (49.2-94.5) | .27 | 1.6 |
| Severity score >6[26] | 60 (32.3-83.7) | 77.3 (54.6-92.2) | 64.3 (35.1-87.2) | 73.9 (51.6-89.7) | .03 | 2.6 |

Castro-Rodriguez stringent predictive index: frequent wheeze plus 1 major or 2 minor criteria (major criteria: parental-doctor diagnosed asthma or doctor-diagnosed eczema in the child; minor criteria: wheezing apart from colds or peripheral blood eosinophilia).[25] Wheeze severity score: 3 to 4 episodes of wheeze plus the number of hospital admissions was used to approximate the score.[26]
*NPV*, Negative predictive value; *PPV*, positive predictive value.
*ASM was measured by using computer-aided analysis as a proportion of the total subepithelial area.

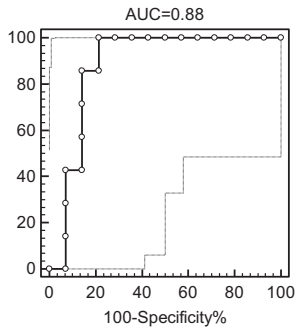


**FIG 3.** Area under the receiver operating characteristics curve *(AUC)* for ASM area fraction in preschool wheezers for the diagnosis of asthma at school age was 0.88 (SE, 0.08; 95% CI, 0.66-0.98; $P < .0001$). CIs are represented by the *broken line*, and the area under the curve is represented by the *continuous line*.

contrast, increased RBM thickness and subepithelial eosinophil values in children with severe recurrent preschool wheeze were not discriminatory and might instead be related to current symptoms rather than future asthma prognosis. We have also shown that preschool ASM mast cells did not predict the development of asthma at school age. Wheeze at preschool and school age (in those who attended for a research visit) was objectively assessed by using a video questionnaire,[13] and subject retention to school age (75%) was comparable with that seen in other studies (54% to 88%).[11,35-38]

We appreciate that the necessarily imposed limitations to our study give rise to the need to interpret our results with caution.

## ASM mass

The proportion of EB specimen ASM in the preschool wheezers was less than reported in older children and adults with asthma.[2,3] The subgroup of children with asthma at school age had 12% (8% to 16%) of the EB specimen subepithelium occupied by ASM, which is similar to the values reported in adults (16% to 18%) with mild-to-moderate asthma.[2,39] Further increases in ASM have been reported with increasing asthma severity, with 16% to 27% of subepithelial tissue occupied by ASM in school-aged children with moderate-to-severe asthma[3,4] compared with 40% in adults with severe asthma.[2] The proportion of ASM occupying the subepithelial tissue in the preschool wheezers and control subjects who did not have asthma was 6%, which is similar to that reported for children aged 6 to 16 years without lower airway disease (4%)[3] but less than that reported in adults with no respiratory symptoms (9% to 11.5%).[2,34]

## ASM mast cells

Although infiltration of ASM by mast cells is associated with increased ASM mass in adults,[8] there was no significant association found between smooth muscle and mast cells in our preschool wheezers. However, we acknowledge that only a relatively small number of the original preschool group (n = 20) had sufficient endobronchial tissue with ASM remaining to stain for smooth muscle mast cells (ie, only 15 who were followed up at school age). Although the numbers of mast cells per ASM area were much higher for both wheezers and control subjects when compared with those seen in adult asthmatic patients, the absolute values for mast cells seen in ASM were low (median, 2 [range, 0-12]) and similar to those reported in adult studies.[8]

## Prediction of future asthma

The area under the ROC curve, which shows the tradeoff between sensitivity and specificity for the capacity of preschool ASM to predict future asthma at school age in preschool wheezers, was high (0.88), but the small numbers had wide CIs. This is comparable with scores for wheeze severity at age 2 years in children from Oslo predicting asthma at 10 years, with an area under the ROC of 0.78; when this score was applied to our

**TABLE IV.** Measurements of lung function, airway inflammation, and atopy at school age in children who attended a research visit

| | Asthma (n = 12) | No asthma (n = 27) | *P* value |
|---|---|---|---|
| Height (cm) | 127.9 (110 to 157) | 135.4 (109 to 146.1) | .6 |
| Height (*z* score) | −0.13 (−3.79 to 2.66) | 0.06 (−3 to 2.16) | .61 |
| Weight (kg) | 24.7 (21.5 to 48) | 26 (16.7 to 37.1) | .64 |
| Weight (*z* score) | 0.26 (−1.66 to 3.13) | 0.35 (−2.07 to 2.81) | .98 |
| | | | |
| Children who completed $FENO_{50}$ | 11 | 22 | |
| $FENO_{50}$ (ppb) | 13.3 (4.9 to 40.3) | 7.08 (2 to 42.6) | **.02** |
| | | | |
| No. of children with SPTs | 12 | 23 | |
| 0 positive SPTs | 4 | 20 | |
| ≥1 positive SPTs | 8 | 3 | **.002** |
| ≥1 positive SPTs, aeroallergen | 7 | 3 | **.01** |
| ≥1 positive SPTs, food allergen | 3 | 0 | |
| | | | |
| No. of children with spirometry | 12 | 23 | |
| $FEV_1$, *z* score | −0.32 (−2.79 to 1.11) | −0.59 (−3.01 to 2.15) | .43 |
| FVC, *z* score | −0.24 (−2.14 to 1.15) | −0.48 (−2.64 to 1.68) | .48 |
| $FEV_1$/FVC ratio, *z* score | 0.105 (−2.39 to 2.55) | −0.4 (−2.75 to 2.15) | .84 |
| | | | |
| No. of children with MBW | 12 | 24 | |
| LCI | 7.33 (6.1 to 10.99) | 7.24 (6.28 to 10.95) | .5 |
| FRC (L) | 1.06 (0.75 to 2.09) | 1.05 (0.71 to 1.61) | .69 |
| | | | |
| No. of children with $S_{cond}$ and $S_{acin}$ | 7 | 22 | |
| $S_{cond}$ | 0.043 (0.003 to 0.06) | 0.014 (−0.027 to 0.03) | **.049** |
| $S_{acin}$ | 0.08 (0.06 to 0.21) | 0.1 (0.028 to 0.62) | .97 |

*FVC*, Forced vital capacity; *LCI*, lung clearance index; *MBW*, multiple-breath washout; $S_{acin}$, acinar airways inhomogeneity.
Data are reported as the median and range. The Mann-Whitney *U* test and Fisher exact test were used, as appropriate. Boldface represents statistically significant results.

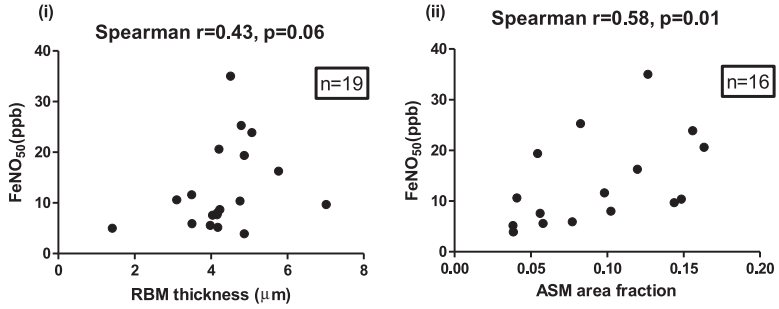


**FIG 4.** Correlation between $FENO_{50}$ values and RBM thickness at school age *(i)* and ASM area fraction *(ii)* in preschool children with severe wheeze. There is a positive correlation between $FENO_{50}$ values and ASM area fraction. There is no correlation between RBM thickness and $FENO_{50}$ values.

preschool wheezers, it resulted in an area under the ROC of 0.70 (see Fig E8 in this article's Online Repository at www.jacionline.org).[26] However, a higher severity score was needed to predict asthma in this group of preschool children compared with the Oslo cohort (>6 vs >5).[26] The median age at follow-up for our study was 8 years; when compared with the Castro-Rodriguez stringent predictive index for asthma at 8 years, an ASM biopsy specimen proportion of greater than 10% had similar negative predictive value (93% vs 88%) but improved positive predictive value (72% vs 44%).[25]

## Subject numbers

The subject numbers were small because only 32 of 42 with ASM in EB specimens could be followed up at school age, and only 8 (25%) of these children had asthma development. However, there was no difference in terms of age at biopsy, sex, or atopy between those who were analyzed at school age and those who were not, such that we do not consider dropouts to have introduced significant bias. To our knowledge, this is the only prospective study in which preschool children had a bronchoscopy and EB at recruitment and have been followed up to school age. Of course, we acknowledge that it would have been informative to repeat EBs at school age to compare airway pathology in preschool and school-aged subjects, but this was ethically impossible in the absence of a clinical indication.

## Severity

The wheezers enrolled in the original study had severe wheeze; otherwise, they would not have undergone bronchoscopy. Thus our data only apply to children with severe preschool wheeze and

cannot be generalized to hose with milder disease. Moreover, the overlap in ASM between children who had childhood asthma and those who did not is too great to be of clinical prognostic value in an individual. However, what this study does suggest is that an early focus on the mechanisms underlying ASM increase might provide a new insight into our understanding of what drives children with severe preschool wheeze to have later asthma.

## Biopsy size

The bronchoscope used to perform biopsies in preschool children was smaller (ie, 2.8 or 3.6 mm) than that used in adults and older children (≥4 mm). The instrument channel was correspondingly smaller (1.2 vs ≥2 mm), and in consequence, the minimum amount of tissue in the biopsy specimen (0.2 $mm^2$) was less than in studies of adults and older children (1 $mm^2$).

## Sample variability

There is variability in structural characteristics between and within EB specimens. The majority of children (40/42) had a single good-quality biopsy specimen for assessment of ASM. For this reason, ASM was analyzed on a per-child basis (the mean of the measurements obtained from all sections from the ≥1 specimens available). However, not all sections from the preschool children that were assessed for ASM were consecutive because sections had also been stained for analyses of other inflammatory cell populations.

## Age

The children who had asthma by school age were older at the time of EB when compared with the children who did not have asthma at school age. Similarly, the children who had asthma at school age had increased endobronchial tissue measured when compared with that seen in control subjects. Data on normal ASM development are scanty. However, there was no relationship between age and ASM in the preschool and school-aged nonwheezing control subjects. A postmortem study has shown that ASM increases linearly with airway diameter during infancy and childhood,[40] but although adults have increased amounts of actual ASM when compared with children, the proportion of ASM in the airway wall remains similar.[41] All biopsy specimens were taken from the third- and fourth-generation peripheral airways in this study.

## Lung function

Lung function testing was not performed at entrance to the study because at the time of the original study (2002-2005), there were no established standards for preschool lung function.[13] However, those children with multiple-trigger wheeze at age 4 to 5 years[42] and asthma at school age had increased ventilation inhomogeneity of the conducting airways. $S_{cond}$ has been shown to be the most sensitive marker of distal airways disease in both preschool children with multiple-trigger wheeze and school-aged children with asthma.[42,43] However, at school age, we had little success with measuring AHR, and the numbers were too small to compare with ASM. $FENO_{50}$, a marker of distal airway inflammation, at 4 to 5 years[42] and 8 years, correlated with ASM area fraction in preschool wheezers. Although there was a positive correlation between subepithelial eosinophil numbers and $FENO_{50}$ values at age 4 to 5 years,[42] none was seen at school age.

## Asthma diagnosis

Defining asthma in young children is difficult.[44] Several children without asthma reported a dry cough at night (n = 7), wheeze (n = 7), and use of inhaled corticosteroids (n = 4), but none of these had ever had a doctor's diagnosis of asthma, and many had a history of upper airway noises or comorbid pathology. Of the children who did not have asthma at school age, 4 had congenital heart disease, 1 had a vascular ring causing some mild airway compression, 1 had ulcerative colitis, 1 had non–cystic fibrosis bronchiectasis, and 1 had a diagnosis of scimitar syndrome at school age. We concede that it is possible we might have misclassified some children as nonasthmatic at school age; however, the increase in $FENO_{50}$ values and the increased likelihood of 1 or more positive SPT responses in the asthma group supports the accuracy of the diagnosis. Seven of the 8 children given a diagnosis of asthma who had ASM measured were still attending tertiary services for asthma management at school age. Longitudinal studies have suggested that children with severe wheeze early in life are more likely to have severe asthma later in life.[45] Six children had persistent asthma symptoms despite treatment with 800 μg of beclomethasone or greater at the time of follow-up, of whom 4 children were treated for severe asthma at school age at the Royal Brompton Hospital (Table I).[46]

Unfortunately, there is no remaining endobronchial tissue in this group of children to explore further the role of ASM in the development of asthma. Although we were unable to characterize the increase in ASM in this group of preschool wheezers, both smooth muscle hypertrophy and hyperplasia contribute to the increase in ASM in adults and school-aged children with asthma.[2,3,39,47] Second, it is likely that ASM cells in asthmatic children are functionally distinct from those in children who do not have asthma. ASM cells express a wide variety of mediators, in particular thymic stromal lymphopoietin and IL-33, both of which are known mediators of airway hyperresponsiveness,[48,49] and it might be that these or other ASM-derived mediators[50] are important in the switch from preschool wheeze to asthma at school age.

In conclusion, we have shown, for the first time, that ASM is increased in those preschool children with severe wheeze who go on to have asthma at school age. The numbers are small and represent children with particularly severe wheeze at preschool age, such that our findings cannot be assumed to be applicable to children with less severe wheeze. However, we consider our data to indicate that future studies exploring the mechanisms underlying the persistence of preschool wheeze and its progression to asthma should focus on ASM.

**Key message**

- **ASM, but not RBM thickness, eosinophilic inflammation, or ASM mast cell numbers discriminate those children with severe recurrent preschool wheeze who go on to have asthma at school age.**

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# METHODS

## Preschool wheezers

All children with severe recurrent wheeze aged 3 months to 5 years had been referred to our tertiary center for further evaluation of symptoms between 2002 and 2005. All children had at least 3 episodes of wheeze (each episode lasting >3 days) in the previous 6 months, and most of the children had at least 1 acute admission to the hospital with wheeze. Wheeze persisted despite trials of bronchodilators and in most children either inhaled corticosteroids, oral corticosteroids, or both. Exclusion criteria were (1) children with isolated cough without associated noisy breathing, (2) children whose main problem was recurrent lower respiratory tract infections, (3) children who were receiving long-term oxygen therapy, and (4) children with chronic lung disease of prematurity. Children were divided into confirmed or reported wheezers based on parental identification of wheeze at preschool age.[E1]

## Nonwheezing preschool control subjects

The majority of nonwheezing control subjects had bronchoscopy for further assessment of upper airway problems, such as stridor, or to assess the degree of compression caused by a vascular ring. Parents provided consent for EBs to be performed for research purposes. Two control subjects with no respiratory symptoms were recruited from patients undergoing a right-sided diagnostic cardiac angiogram. Parental consent was obtained to perform bronchoscopy and research biopsy before angiography.

## Fiberoptic bronchoscopy and EB

Fiberoptic bronchoscopy and EB were performed after achievement of general anesthesia by using a 2.8-mm or 3.6-mm bronchoscope (Olympus KeyMed, Southend on Sea, United Kingdom) with a 1.2-mm channel, allowing 1-mm rat-tooth forceps (Olympus Key Med, serial no. FB-56D-1) to be used. Up to 4 EB specimens were taken from the third- or fourth-generation subsegmental bronchi of the right lower lobe. Full blood counts, IgE levels, and specific RAST results (milk, egg, peanut, house dust mite, cat, dog, and grass pollens) were obtained from a blood sample taken while the child was anesthetized.

## Recruitment at school age

The study was approved by the local research ethics committee, and all procedures were performed with informed parental consent and child assent, where appropriate.

## Spirometry and exhaled nitric oxide

For many children, it was their first attempt at spirometry and measurement of $\text{FENO}_{50}$, and a period of training was necessary. $\text{FENO}_{50}$ values were measured before spirometry.[E2] For the measurement, nitric oxide–free air was inhaled to near-total lung capacity over a period of 2 to 3 seconds through the mouthpiece of the instrument. The exhaled nitric oxide value is the mean nitric oxide level during a 3-second nitric oxide plateau. Three acceptable readings after a period of practice (defined as agreement to within 10%) were performed with at least 30-second intervals between maneuvers. $\text{FENO}_{50}$ values were calculated as the mean of the plateaus from 3 technically satisfactory exhalations. Spirometry was performed with a Vitalograph Compact (Vitalograph, Buckingham, United Kingdom). The highest sum of forced vital capacity and $\text{FEV}_1$ of the 3 maneuvers was recorded as per American Thoracic Society/European Respiratory Society guidelines.[E3] All spirometric results were compared with appropriate recent reference ranges and analyzed as $z$ scores.[E4]

## Clinical history at school age

The study questionnaire used was the International Study of Asthma and Allergy in Children core questionnaire for asthma, eczema, and allergic rhinitis.[E5] A second questionnaire recorded medication history, including oral and inhaled corticosteroid use; total number of hospital admissions with wheeze; presence of household pets; length of time spent in the nursery; and parental smoking. Parents were also asked whether their child's symptoms were mainly associated with colds or allergy or whether they were triggered by multiple stimuli. Although information, such as family history of asthma, birth details, and parental smoking during pregnancy, had previously been recorded, the answers were rechecked. Both questionnaires were interviewer directed, and the answers were recorded by the interviewer. Current eczema was defined as a positive response to both of the following questions: "Has your child ever had an itchy rash which was coming and going for at least 6 months?" and "Has your child had this itchy rash at any time in the last 12 months?" Current allergic rhinitis was defined as a positive response to the following question: "In the past 12 months, has your child had a problem with sneezing, or a runny, or a blocked nose when he/she did not have a cold or the flu?"

## Video questionnaire at school age

Parents were shown a modified video questionnaire with 4 clips: (1) wheeze (male, 6 years), (2) nasal upper respiratory noises (female, 10 months), (3) wet cough (male, 7 years), and (4) stridor (male, 4 years). Video clips 1 and 2 were shown at preschool and school age. The third and fourth video clips were changed from those used at preschool age, which showed children aged 6 and 10 months, to more age-appropriate examples of stridor and wet cough. All video clips had been previously validated.[E6] Parents were again asked "Which if any of these noises does your child make?" They were also asked the following: "Has your child made any of these noises in the past?" They were free to choose 1 or more video clips.

## School-aged nonwheezing control subjects

To assess the relationship between age and ASM, school-aged children (n = 13; age, 5.9-14.2 years) had ASM measured by using point and line intersection counting (by C.J.B.). Children had clinically indicated bronchoscopy for evaluation of recurrent croup or a barking cough (n = 6), noisy breathing (n = 1), stridor (n = 2), and shortness of breath (n = 1).[E7] Three children had bronchoscopy for investigation of hemoptysis but had normal anatomic appearances on bronchoscopy. All parents consented to EB for research purposes. EB specimens were processed and stained in an identical manner to the preschool EB specimens.

## Asthma predictive indexes

Clinical data at preschool age were used to classify preschool wheezers by using the Castro-Rodriguez stringent clinical predictive index of frequent wheeze plus 1 major or 2 minor criteria (major criteria: parental doctor-diagnosed asthma or doctor-diagnosed eczema in the child; minor criteria: wheezing apart from colds or peripheral blood eosinophilia).[E8] Frequent wheeze for this study was defined as a score of greater than 3 at both the year 2 and year 3 wheeze surveys (scale, 1-5; from "very rarely" to "on most days"). A history of doctor-diagnosed allergic rhinitis had not been recorded, and therefore wheezing apart from colds or peripheral blood eosinophilia was used as the minor criteria. All children had at least 3 episodes of wheeze at the time of the EB; however, the exact number was not recorded. For application of the wheeze severity score, 3 to 4 episodes of wheeze plus the number of hospital admissions was used to approximate the score.[E9] Both clinical predictive indexes were compared with current asthma at school age.

## Biopsy specimen processing and immunostaining

Up to 4 EB specimens were immersed in 10% formal saline and processed to paraffin blocks between 4 and 24 hours after fixation. Five-micrometer-thick sections were taken at 25- to 50-μm step intervals depending on the biopsy specimen size and were stained with hematoxylin and eosin to assess RBM thickness and ASM. Monoclonal antibody was applied, which had been raised from mice against human intracellular isotopes to sections, for eosinophil protein X (EG2; Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) and mast cells (mast cell tryptase; clone AA1, Dako, Glostrup, Denmark).

## Evaluable biopsy specimens

Evaluable biopsy specimens were those fulfilling the following criteria: (1) presence of epithelium, RBM, and associated subepithelial tissue and (2) minimal crush artifact, edema, or blood within the biopsy specimen.

## Measurement of RBM thickness and subepithelial eosinophils

RBM thickness and subepithelial eosinophils were remeasured while blinded to the children's clinical history (by R.O.). The geometric mean of 40 measurements taken 20 μm along the RBM was used to represent the mean thickness for that biopsy specimen.[E1,E10] Volume density of subepithelial eosinophils was measured by using point counting.[E1] Areas of smooth muscle, vessels, and glands were excluded.

## Measurement of ASM

ASM was measured in 2 ways, the first using computer-aided image analysis and the second using stereologic techniques.

**Method 1.** ASM area and total subepithelial area were measured at a ×100 magnification by drawing around the outer limits of the smooth muscle bundles and the subepithelial area of the biopsy specimen (Fig E1). The subepithelial and ASM area was then calculated by using computer-aided image analysis. ASM was then expressed as a fraction of the total subepithelial area.

**Method 2.** ASM volume fraction was also measured by using a stereologic technique, namely point and line intersection counting. A Weibel (M168) grid was placed on the biopsy specimen at random and moved systematically until the whole specimen was measured (Fig E2). ASM volume fraction was measured at ×200 magnification and expressed as a fraction of the total number of points falling on smooth muscle over the total number of points falling on the subepithelium. The formula used to calculate this was as follows:

$$\mathrm{Vv(sm/subepithelium)} = \left(\sum \text{points on ASM}\right) / \left(\sum \text{points on subepithelial tissue}\right).$$

ASM volume fraction was also indexed to the surface area of the RBM by counting the number of lines intersecting the apical surface of the RBM. The following formula was used:

Volume fraction of ASM indexed to surface area of RBM:

$$\mathrm{V/S\ (sm/rbm)} = \left(\sum \text{points on ASM} \times 1[\mathrm{p}]\right) / \left(2 \times \sum \text{line intersections with RBM}\right),$$

where *l(p)* denotes the length per point (68 μm, Fig E9).[E11]

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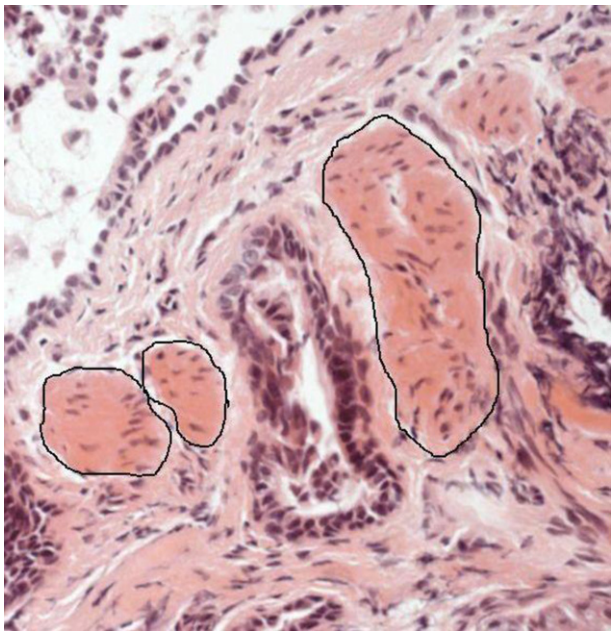

**FIG E1.** Smooth muscle bundles were traced with a computer mouse, and then the area was calculated by using computer-aided analysis. ASM was represented as a fraction of the total subepithelial area (scale not shown: image shown for illustration purposes).

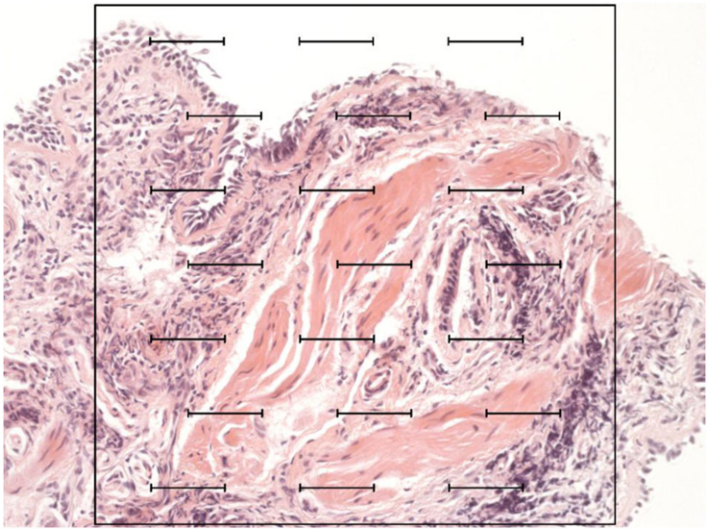

**FIG E2.** Smooth muscle volume fraction was quantified by using point and line intersection counting. A Weibel grid (M168) was overlaid at the section, and ASM was measured at ×200 magnification. The length between 2 points was 68 μm.

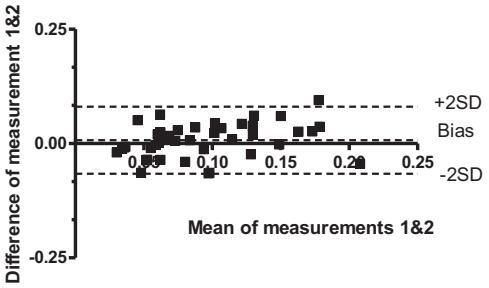


**FIG E3.** A Bland-Altman plot of ASM area fraction measurements between observer 1 and observer 2. Good repeatability between ASM area fraction measurements was shown between observer 1 and observer 2.

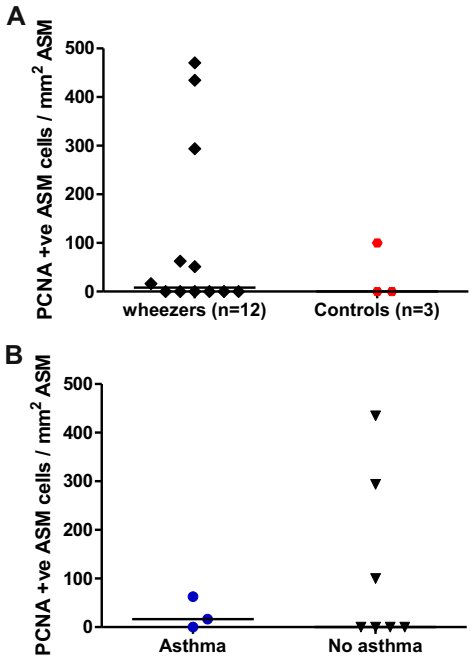


**FIG E4.** **A**, No difference in smooth muscle proliferation between wheezers and control subjects. **B**, No difference in smooth muscle proliferation between children with and without asthma at school age. Smooth muscle proliferation was assessed by quantifying the proportion of proliferating cell nuclear antigen *(PCNA)*–positive smooth muscle cells per square millimeter of smooth muscle.

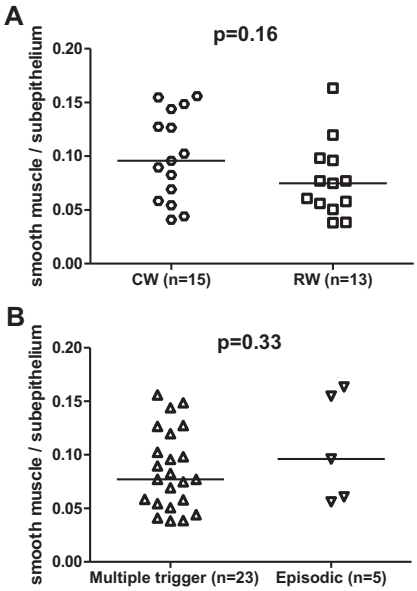


**FIG E5.** There was no difference between ASM area fraction in confirmed *(CW)* and reported *(RW)* wheezers **(A)** or in multiple-trigger or episodic (viral) wheezers **(B)** at preschool age. The Mann-Whitney *U* test was used.

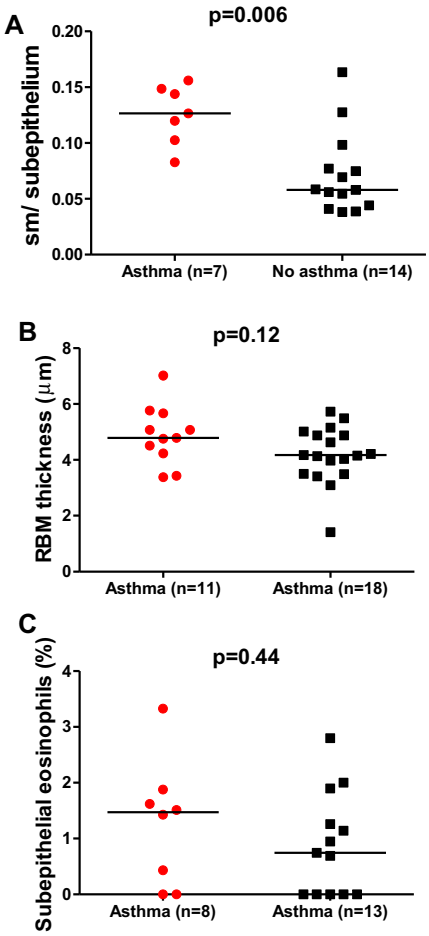


**FIG E6.** ASM area fraction is increased in preschool wheezers with asthma at school age **(A)**, but there was no difference in RBM thickness **(B)** or subepithelial eosinophil numbers **(C)** between preschool wheezers who did and did not have asthma at school age. The Mann-Whitney *U* test was used.

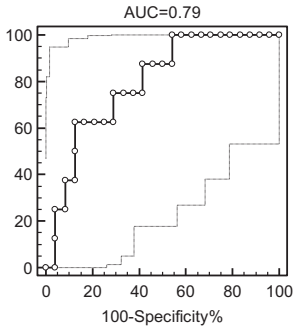


**FIG E7.** Receiver operating characteristics curve for ASM volume fraction measured by using point and line intersection counting in preschool wheezers related to asthma at school age (area under the curve *[AUC]*, 0.79; SE, 0.08; 95% CI, 0.61-0.91; $P = .0008$). CIs are represented by the *broken line*, and the area under the curve was represented by the *continuous line*.

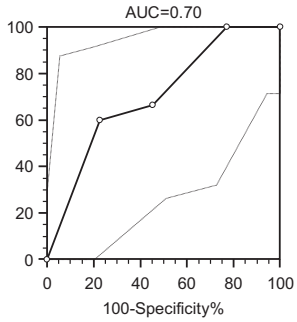


**FIG E8.** Receiver operating characteristics curve for wheeze severity score related to asthma at school age (area under the curve *[AUC]*, 0.7; SE, 0.08; 95% CI, 0.53-0.84; $P = .01$) in preschool wheezers recruited from the Royal Brompton Hospital from 2002-2005. CIs are represented by the *broken line*, and the area under the curve was represented by the *continuous line*.

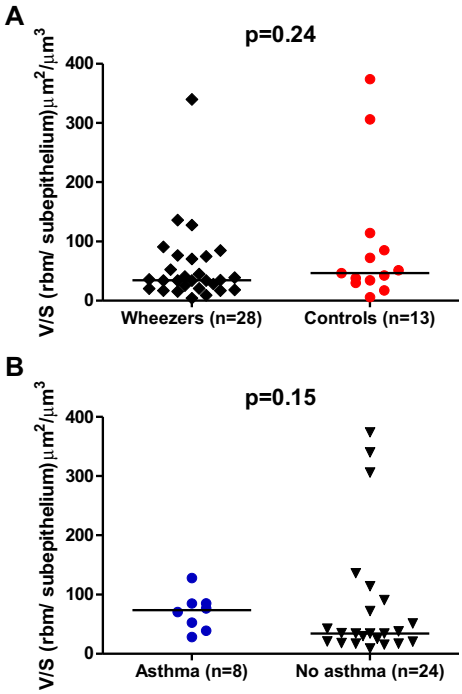


**FIG E9.** No difference in volume fraction of ASM indexed to surface area of RBM between preschool wheezers and control subjects **(A)** and children with and without asthma at school age **(B)**. The Mann-Whitney *U* test was used. *V/S,* Volume fraction of smooth muscle indexed to surface area of RBM.

**TABLE E1.** Comparison of clinical characteristics of preschool children followed up at school age and those lost to follow-up

| | Followed up (n = 51) | Lost to follow-up (n = 17) | *P* value |
|---|---|---|---|
| Age at biopsy (mo)* | 19 (3-57) | 15 (7-58) | .77 |
| Male/female sex | 32/19 | 12/5 | .77 |
| Wheezers/control subjects | 37/14 | 10/7 | .36 |
| Preschool IgE (IU/mL) | 21 (1-635)† | 18 (1-2605) | .99 |

*Data are reported as the median and range. The Mann-Whitney *U* test was used.
†Results not available for 5 children.

**TABLE E2.** Comparison of clinical characteristics of children with and without ASM on evaluable EB specimens

| | ASM (n = 49) | No ASM (n = 7) | *P* value |
|---|---|---|---|
| Age at biopsy (mo)* | 19 (3-58) | 13 (6-55) | .39 |
| Male/female sex | 31/18 | 3/4 | .42 |
| Wheezers/control subjects | 34/15 | 4/3 | .67 |
| Preschool IgE (IU/mL) | 16 (1-635)† | 17 (2-559) | .89 |

*Data are reported as the median and range. The Mann-Whitney *U* test was used.
†Results not available for 3 children.

**TABLE E3.** Comparison of clinical characteristics of children followed up at school age with ASM on EB and those who were either lost to follow-up or did not undergo ASM on EB

| | Followed up (n = 32) | Lost to follow-up (n = 36) | *P* value |
|---|---|---|---|
| Age at biopsy (mo)* | 18.5 (3-57) | 20 (3-58) | .5 |
| Male/female sex | 19/13 | 25/11 | .45 |
| Wheezers/control subjects | 21/11 | 26/10 | .6 |
| Preschool IgE (IU/mL) | 28.5 (1-635)† | 17 (1-2605)‡ | .99 |

*Data are reported as the median and range. The Mann-Whitney *U* test was used.
†Results not available for 2 children.
‡Results not available for 3 children.

**TABLE E4.** Comparison of clinical characteristics of children followed up at school age by research visit and by telephone questionnaire

| | Research visit (n = 39) | Telephone questionnaire (n = 12) | *P* value |
|---|---|---|---|
| Age at biopsy (mo)* | 18 (3-57) | 32.5 (3-55) | .36 |
| Male/female sex | 14/15 | 8/4 | .32 |
| Wheezers/control subjects | 27/12 | 10/2 | .47 |
| Preschool IgE (IU/mL) | 21 (1-635)‡ | 27 (1-559)† | .75 |

*Data are reported as the median and range. The Mann-Whitney *U* test was used.
†Results not available for 2 children.
‡Results not available for 3 children.

**TABLE E5.** No correlation between ASM area fraction (measured using computer aided analysis) or ASM volume fraction (measured by using point and line intersection counting) and RBM thickness and subepithelial eosinophil or smooth muscle mast cell values

| | | ASM area fraction | | ASM volume fraction | |
|---|---|---|---|---|---|
| | No | Spearman *r* | *P* value | Spearman *r* | *P* value |
| Subepithelial eosinophils (%) | 32 | −0.05 | .75 | −0.12 | .49 |
| RBM (μm) | 40 | 0.2 | .19 | 0.19 | .23 |
| Smooth muscle mast cells/mm$^2$ | 20 | −0.01 | .94 | −0.03 | .86 |