

Ciliary dysfunction and ultrastructural abnormalities are features of severe asthma

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Background: Epithelial dysfunction has been implicated in asthma pathophysiology, but no studies have directly assessed ciliary function in asthma.

Objective: To study the ciliary function and epithelial ultrastructure of patients with asthma and healthy controls.

Methods: We studied ciliary beat frequency and beat pattern by using digital high-speed video imaging and ultrastructure by transmission electron microscopy of bronchial epithelial strips from 7 subjects with mild, 7 with moderate, and 19 with severe asthma and 9 healthy controls.

Results: The median (interquartile range) ciliary beat frequency was decreased in moderate (6.5 [4.4-8.5] Hz) and severe asthma (6.7 [6.1-7.6] Hz) compared with controls (10.5 [9.7-11.8] Hz; $P < .01$). Dyskinesia and immotility indices were higher in severe asthma (65% [43%-75%]; 6.3% [1%-9.5%], respectively) compared with controls (4% [0%-6.7%]; 0%, respectively; $P < .01$). These abnormalities were related to disease severity (ciliary beat frequency, $r_s = -0.68$; dyskinesia index, $r_s = 0.86$; immotility index, $r_s = 0.65$; $P < .0001$). The ultrastructure of the epithelium was abnormal in severe asthma with a reduction in ciliated cells, an increase in dead cells, and ciliary disorientation compared with all other groups ($P < .05$). Compared with patients with mild asthma and healthy controls, patients with severe asthma showed increased ciliary depletion, microtubular defects, mitochondrial damage, and cytoplasmic blebbing ($P < .01$). All of these changes were related to disease severity.

Conclusion: Ciliary dysfunction and ultrastructural abnormalities are closely related to asthma severity. Ciliary dysfunction is a feature of moderate to severe asthma, and profound ultrastructural abnormalities are restricted to severe disease. Whether these changes contribute to the development of

severe asthma phenotype remains to be determined. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Ciliated epithelium, ciliary beat frequency, ciliary beat pattern, ciliary disorientation, ciliary dyskinesia, refractory asthma, severe asthma

Asthma affects approximately 300 million people globally.¹ Although the majority of patients have well controlled disease, there remains a subgroup of adults with asthma, accounting for about 10% of patients with asthma, who continue to have debilitating chronic and persistent symptoms despite optimal standard asthma treatment.² This group with severe refractory asthma³ represents those with a high risk of severe exacerbations and asthma-related mortality and accounts for >50% of asthma-related health care costs.^{4,5} There has been extensive research to unravel the complex pathophysiology of severe asthma, but it remains uncertain what the immunopathologic hallmarks of severe asthma are.^{2,5} In this respect, the role of the dysfunctional respiratory epithelium in severe asthma has been of much recent interest.^{6,7}

The ciliated epithelium that covers the surface of the airways forms an immunologically active natural barrier to invasion and injury by inhaled pathogenic organisms and particulate material. The epithelium is lined by an airway surface liquid and the mucus layer. The airway surface liquid provides an ideal environment in which the cilia beat at a frequency of 11 to 14 Hz. The mucus layer that lies above the airway surface liquid is cleared from the airway by the highly coordinated ciliary beating. This process, known as mucociliary clearance,⁸ is an essential factor in pulmonary defense.⁹ It therefore follows that damage to the respiratory epithelium and ciliary dysfunction causes impaired mucociliary clearance, leading to increased susceptibility to infection and inflammation.

Using an inhaled radio-aerosol technique, mucociliary clearance in asthma in the stable state and during exacerbations was reported to be impaired.^{10,11} Optimal mucociliary clearance depends on the structural and functional integrity of the cilia as well as the characteristics of the airway surface liquid and mucus. Submucosal gland hypertrophy and goblet cell hyperplasia are well recognized pathologic features of asthma.¹² The asthmatic airway is also characterized by mucus hypersecretion,¹³ abnormal mucus rheology,¹² and tethering of intraluminal mucins to goblet cells in the airway epithelium.¹⁴ Although evidence that supports abnormalities of mucus¹²⁻¹⁴ and airway surface liquid¹⁵ provides a plausible mechanistic explanation for the reduced mucociliary clearance observed in asthma, to date no studies have assessed ciliary function directly in asthma or considered its relationship with disease severity. Indeed, impaired ciliary function would have a pronounced effect on mucociliary clearance. In this context, in

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Abbreviations used

FVC: Forced vital capacity

GINA: Global Initiative for Asthma

IQR: Interquartile range

this observational study, we aimed to characterize the ciliated respiratory epithelium from the lower airways of adults with mild, moderate, and severe asthma compared with healthy controls by assessing the function of cilia and detailed ultrastructure of the ciliated epithelium.

METHODS

Subjects

Patients with mild ($n = 7$), moderate ($n = 7$), and severe ($n = 19$) asthma were recruited from clinics at Glenfield Hospital, Leicester, United Kingdom, over a 2-year period (2007–2009). Healthy control subjects ($n = 9$) were recruited from hospital staff and by local advertising. Asthma was diagnosed on the basis of the presence of clinical features consistent with asthma and objective measures of airway hyperresponsiveness and variable airflow obstruction (PC_{20} in FEV_1 of less than 8 mg/mL; increase in FEV_1 by at least 15% after the inhalation of 200 μ g salbutamol; or variation in peak flow, expressed as a percentage of the mean, exceeding 20% over a period of 14 days). Asthma severity was classified as mild, moderate, and severe by using the current Global Initiative for Asthma (GINA) guidelines on the basis of the GINA treatment steps¹⁶ (mild asthma, GINA treatment step 1/2; moderate asthma, GINA treatment step 3; severe asthma, GINA treatment step 4/5). Patients with severe asthma also met the American Thoracic Society criteria for refractory asthma.³ At the time of collection of bronchial epithelial samples, patients with asthma had been free from intercurrent respiratory infections and asthma exacerbations requiring antibiotics and/or rescue use of systemic corticosteroids for at least 6 weeks. Healthy subjects had no history of respiratory disease, normal lung function, and normal PC_{20} . All subjects were current nonsmokers, and those who did smoke in the past had a smoking history of less than 10 pack-years. The study protocol was approved by the Leicester-shire and Rutland regional ethics committee, and written informed consent was obtained from all subjects.

Measurements

Demographic and medical details including age, sex, and current medications were recorded on all subjects. FEV_1 , forced vital capacity (FVC), and FEV_1 /FVC ratio were measured on all subjects with a rolling-seal spirometer (Vitalograph Ltd, Buckingham, England). The PC_{20} was computed from the methacholine dose-response curve (the change in FEV_1 in relation to the methacholine concentration) by linear interpolation on a log scale using standard techniques.¹⁷ In patients with asthma, the presence of atopy was determined by allergen skin prick tests for common aeroallergens including *Dermaphagoides pteronyssinus*, dog, cat, and grass pollen. Plasma IgE was measured on patients with moderate and severe asthma. Patients with asthma had single-flow exhaled nitric oxide concentration measured at a rate of 50 mL per second as previously described.¹⁸ Induced sputum was obtained from patients with asthma, and sputum samples were processed as previously described.¹⁹ All subjects underwent flexible bronchoscopy conducted according to the British Thoracic Society guidelines²⁰ to obtain strips of bronchial epithelium by brushing the bronchus intermedius.

Ciliary beat frequency and beat pattern

Detailed methodology is given in this article's Methods in the Online Repository at www.jacionline.org. Typically, a strip of bronchial epithelium obtained by brushing the bronchus contained a row of adjacent bronchial epithelial cells. Ciliary beat frequency was measured and beat pattern assessed

on strips of bronchial epithelium by using a digital high-speed video microscopy system as described previously with nasal epithelial brushings, within 2 hours of sample collection.^{21,22} The high-speed video images were analyzed in a blind fashion. The images were re-analyzed by a second observer (A.R.) and blinded on a second occasion by the original observer (B.T.).

The experimental system allowed the ciliary beat pattern to be evaluated in 3 different planes: sideways profile of the ciliated epithelial strip, assessing the ciliated epithelial strip with cilia beating directly toward the observer, and assessing the ciliated epithelial strip from directly above.^{21,22} The path taken by a cilium during the beat cycle was analyzed frame by frame. This was characterized and compared with the normal beat pattern²¹ seen on digital high-speed video analysis. Dyskinesia was defined as an abnormal beat pattern that included reduced beat amplitude, stiff beat pattern, failure to bend along the length of the ciliary shaft, a flickering or twitching motion, and static cilia. The dyskinesia index was calculated as the percentage of dyskinetic cilia within the sample (number of dyskinetic readings/total number of readings for sample $\times$ 100). The immotility index²³ was calculated as the percentage of immotile cilia within the sample (number of immotile readings/total number of readings for sample $\times$ 100).

Transmission electron microscopy

The detailed ultrastructure of the bronchial epithelial strips was studied by using transmission electron microscopy as described before with nasal epithelial brushings.²⁴ Detailed methodology is given in the Methods in the Online Repository. The ciliated epithelium was assessed in a blind fashion for both epithelial and ciliary ultrastructural changes. Epithelial integrity was assessed first by assessing the cell type. The number of ciliated cells, unciliated cells, mucus cells, and dead cells were expressed as a percentage of all cells examined. Disruption and damage to the epithelium was assessed by calculating the percentages of ciliated cells with loss of cilia, cellular projections, cytoplasmic blebbing, and mitochondrial damage among all cells examined. Damage to individual cilium was evaluated by examining ciliary ultrastructure for microtubular and dynein arm defects, and the percentage of cilia with microtubular or dynein arm defects was calculated. Intracellular ciliary orientation, defined as the standard deviation of the angles of lines through the central pair of microtubules of cilia originating from a single ciliated cell, was determined as described previously.²⁵

Statistical analysis

Sample size was calculated on the basis of ciliary beat frequency as the primary outcome measure. Seybold et al²⁶ studied the surface liquid velocity on freshly excised sheep trachea and found that a 16% increase in ciliary beat frequency correlates with a 56% increase in the tracheal surface liquid velocity. Hence, we assumed that an absolute mean difference in ciliary beat frequency of 2 Hz has potential biological significance. It was estimated that, to detect a mean difference in ciliary beat frequency of 2 Hz (with a SD of 1 Hz) between 2 groups with a CI of 95% and a power of 80%, a sample size of 6 ($n = 6$) in each group would be required. Statistical analysis was performed by using GraphPad Prism 5 (GraphPad software Inc, Calif) and SAS/STAT software (SAS Institute Inc, NC). Nonparametric data were described as medians (interquartile ranges [IQRs]). Groups were initially compared by using the Kruskal-Wallis test, and *post hoc* analysis was performed by using the Dunn method. The Spearman correlation was used to assess the univariable relationship between disease severity and abnormalities of ciliary function and epithelial ultrastructure. A P value of $<.05$ was taken as the threshold for statistical significance in each case. Agreement between the 2 observers was excellent for measurement of ciliary beat frequency (interclass correlation, 0.94) as well as dyskinesia index (interclass correlation, 0.93). Repeatability (agreement within observer) was also excellent (interclass correlation was 0.94 for ciliary beat frequency and 0.99 for dyskinesia index).

RESULTS

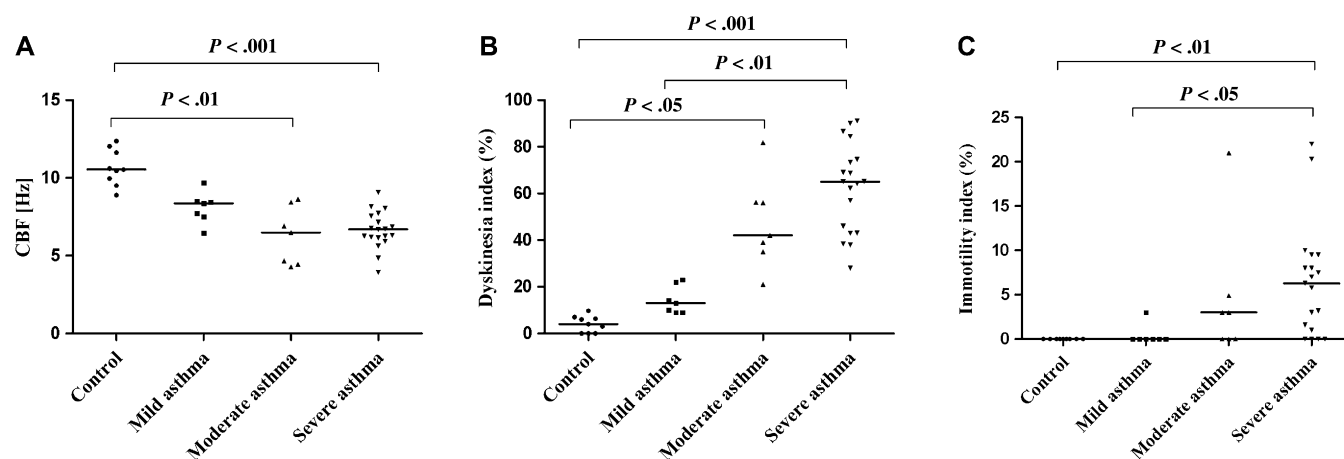
The baseline characteristics of the subjects are given in Table I. Ciliary beat frequency was decreased in moderate to severe

TABLE I. Baseline characteristics of the subjects

Characteristic	Control (n = 9)	Mild asthma (n = 7)	Moderate asthma (n = 7)	Severe asthma (n = 19)
Age (y)*	35 (22.5-52.5)	47 (39-60)	45 (42-56)	42 (38-48)
Sex (no.), male/female	4/5	2/5	3/4	8/11
Age at onset of symptoms (y)*	—	39 (20-49)	36 (21-47)	12 (3-28)
Atopy (n)	—	4	4	11
Plasma IgE (IU/mL)*	—	—	71.1 (34.7-406.5)	131 (73.4-356)
FEV ₁ (L)*	3.7 (3.2-4.1)	2.2 (2.2-2.7)	2.3 (2.0-3.1)	2.4 (1.8-2.9)
FEV ₁ percentage of predicted value	102 (97-109)	80 (70-92)	77 (71-107)	76 (64-92)†
FEV ₁ : FVC ratio (%)*	91 (85-95)	72 (68-77)	71 (69-78)	72 (65-78)†
Exhaled nitric oxide (ppb)*	—	0.9 (0-4.9)	14 (6.6-25.7)	46.8 (15.9-99)‡
Sputum measurements*				
Eosinophils (%)	—	0.5 (0-1.1)	1.8 (1-3.5)	4.0 (0.7-32)
Neutrophils (%)	—	32 (13-58)	60 (50-68)	59 (43-86)
PC ₂₀ (mg methacholine/mL)*	>16	0.6 (0.2-5.8)	0.2 (0.2-0.9)	0.3 (0.06-1.2)
Inhaled steroid dose in µg (BDP equivalent)*	0	400 (400-800)	800 (800-1000)	1600 (1600-2000)‡§
No. of subjects on continuous oral steroids	0	0	0	7
No. of subjects on long-acting bronchodilator	0	0	7	19

BDP, Beclomethasone dipropionate; —, not applicable.

*Data expressed as medians (IQRs).

† $P < .01$ compared with control group.‡ $P < .01$ compared with mild asthma group.§ $P < .01$ compared with moderate asthma group.**FIG 1.** Ciliary beat frequency (CBF, **A**), dyskinesia index (**B**), and immotility index (**C**) of healthy controls and patients with mild, moderate, and severe asthma.

asthma. The median (IQR) ciliary beat frequency was decreased in moderate (6.5 [4.4-8.5] Hz) and severe asthma (6.7 [6.1-7.6] Hz) compared with controls (10.5 [9.7-11.8] Hz; Kruskal-Wallis $P < .01$; $P < .001$ between groups; Fig 1, A). Analysis of ciliary beat pattern showed a higher proportion of dyskinetic and immotile cilia in moderate to severe asthma. The median (IQR) dyskinesia index was increased in moderate (42% [35%-56.1%]) and severe asthma (65% [43.1%-74.6%]) compared with controls (4% [0%-6.7%]; Kruskal-Wallis $P < .05$; $P < .001$ between groups; Fig 1, B) and was increased in severe versus mild asthma (13% [9%-22%]; Kruskal-Wallis $P < .01$; Fig 1, B). The median (IQR) immotility index was increased in severe asthma (6.3% [1.0%-9.5%]) compared with mild asthma (0%) and controls (0%; Kruskal-Wallis $P < .05$; $P < .01$ between groups; Fig 1, C). All of these abnormalities were related to

disease severity (see this article's Table E1 in the Online Repository at www.jacionline.org). The median (IQR) length of cilia was 6 (6-6) µm for healthy subjects, 6 (5.5-6.0) µm for patients with mild asthma, 6 (6-6.25) µm for patients with moderate asthma, and 6 (4.5-6.25) µm for patients with severe asthma, and the difference between the groups was not significant ($P = .89$). Examples of digital high-speed video recordings of ciliated edges of patients with severe refractory asthma and healthy controls are provided in the Online Repository (see this article's Videos E1 and E2 at www.jacionline.org).

Ten of 19 patients with severe asthma had eosinophilic asthma (sputum eosinophils > 3%). In the severe asthma group, the median (IQR) ciliary beat frequencies of those with eosinophilic asthma and noneosinophilic asthma were 7.3 (6.1-8.1) Hz and 6.3 (5.5-6.8) Hz, respectively, and the difference was not statistically

TABLE II. Analysis of epithelial ultrastructure by transmission electron microscopy†

Ultrastructural parameter	Control (n = 7)	Mild (n = 6)	Moderate (n = 6)	Severe (n = 12)	Spearman <i>r</i> (between asthma severity and ultrastructural features) and <i>P</i> value
Ciliated cells	83.6 (80.6-87.7)*	85.8 (81.4-87.7)*	86.9 (72.7-90.1)*	70.6 (61.3-71.8)	-0.62, <i>P</i> = .0002
Unciliated cells	11.4 (9.3-15.7)	10.6 (7.7-14.2)	9.1 (7.3-13.1)	14.2 (12.7-15.2)	0.23, <i>P</i> = .21
Mucus cells	3.4 (2.9-3.8)**	4.4 (3.7-5.2)	4 (2.6-7.2)	7.9 (5.3-10.2)	0.66, <i>P</i> < .0001
Dead cells	0 (0-1)**	0 (0-0.3)**	0 (0-5.7)*	8.3 (5.8-13.5)	0.71, <i>P</i> < .0001
Dynein arm defects	0.9 (0.6-1.9)	1.6 (1.2-2.8)	2.9 (2.8-3.6)	3.1 (1.3-6.5)	0.45, <i>P</i> = .012
Microtubular defects	1.6 (1.2-2.0)**	1.4 (1.2-2.1)**	2.7 (1.6-6.2)	5.7 (2.5-11)	0.69, <i>P</i> < .0001
Ciliary orientation (degrees)	13.5 (12.7-14.1)***	14 (12.7-14.9)*	14 (12.9-15.9)*	20.7 (17.5-23.2)	0.79, <i>P</i> < .0001
Ciliated cells with loss of cilia	13.5 (9.7-14.9)**	13.9 (10.3-16.9)**	17.5 (11.5-21.8)	39 (29.3-51.5)	0.76, <i>P</i> < .0001
Cells extruding from the surface	12.9 (8.8-18.4)***	17.7 (13-23.8)**	24.4 (19.1-26.9)	44.7 (36.6-54.8)	0.89, <i>P</i> < .0001
Cells with cytoplasmic blebbing	13.7 (7.7-14.9)**	10.6 (8.9-14.4)***	19.3 (15.2-25.5)	43.6 (27.3-49.3)	0.77, <i>P</i> < .0001
Cells with mitochondrial damage	8.7 (3.7-11.4)**	9.8 (5.6-13)**	11.7 (9.1-24.0)	35 (31.5-46.4)	0.76, <i>P</i> < .0001

****P* < .001.***P* < .01.**P* < .05 compared with severe asthma group.

†Data expressed as median percentages (IQRs). Ciliary orientation is expressed as median degrees (IQRs).

significant (*P* = .12). Similarly, there was no significant correlation between sputum eosinophilia and ciliary beat frequency ($r_s = 0.38$; *P* = .11), dyskinesia index ($r_s = -0.13$; *P* = .59), or immotility index ($r_s = 0.17$; *P* = .48), in the severe asthma group. We also examined correlation between ciliary functional characteristics and duration of asthma, FEV₁, FVC, exhaled nitric oxide (eNO), IgE, PC₂₀, and proportion of sputum neutrophils. We found that FEV₁ and PC₂₀ correlate significantly with ciliary beat frequency ($r_s = 0.32$, *P* < .05; $r_s = 0.47$, *P* < .01, respectively) and the dyskinesia index ($r_s = -0.42$, *P* < .01; $r_s = -0.50$, *P* < .01, respectively).

Bronchial epithelium obtained from 2 control subjects, 1 patient with mild asthma, 1 patient with moderate asthma, and 7 patients with severe asthma were insufficient for assessment by transmission electron microscopy. Therefore, transmission electron microscopy was done on samples obtained from 7 control subjects, 6 subjects with mild asthma, 6 subjects with moderate asthma, and 12 subjects with severe asthma. The median (IQR) no of epithelial cells per sample studied was 203.5 (162.8-263.3; range, 106-359). Results are summarized in Table II, and example electron micrographs are shown in Figs 2 and 3. The ultrastructure of the ciliated epithelium was abnormal in severe asthma with a reduction in ciliated cells, an increase in dead cells, and ciliary disorientation compared with all of the other groups (*P* < .05); with ciliary depletion, microtubular defects, mitochondrial damage, cytoplasmic blebbing, and loss of epithelial integrity compared with patients with mild asthma and healthy controls alone (*P* < .01); and with an increase in mucus cells compared with healthy controls only (*P* < .01). There were no significant differences across groups for the proportion of unciliated cells or dynein arm defects. All of the ultrastructural changes, except the proportion of unciliated cells, were related to disease severity (Table II).

DISCUSSION

These data provide evidence that ciliary dysfunction and ultrastructural abnormalities are closely related to asthma severity, with dysfunction a feature of moderate to severe asthma and profound ultrastructural abnormalities restricted to severe disease. These striking changes, particularly in those subjects with

severe asthma, constitute a phenotype of profound secondary ciliary dysfunction and are likely to have important functional consequences.

To our knowledge, this is the first study that has assessed ciliary function in terms of ciliary beat frequency and beat pattern in a well characterized group of patients with asthma of varying severity. We used bronchial epithelial brushings rather than biopsies because, in our experience, we have found that ciliary function (ciliary beat frequency and beat pattern) studies are technically much easier to perform on epithelial brushings, and this is an accepted method for studying ciliary function.²⁵ Our study is unique in that, in addition to showing a markedly reduced ciliary beat frequency, we determined ciliary beat pattern, which was also found to be significantly abnormal in patients with moderate and severe asthma. This was made possible by the recent advent of high-resolution digital high-speed video imaging²¹ that allowed us to assess the precise beat pattern of cilia by viewing the ciliary beat cycle frame by frame in different planes. Using this method, we have previously shown that cilia in certain conditions may have a normal beat frequency despite markedly abnormal beat pattern,²⁴ and normal ciliary beat frequency does not necessarily equate to normal ciliary function. The marked reduction in ciliary beat frequency and abnormal beat pattern seen in asthmatic airway epithelium, particularly in those with severe disease, is likely to be multifactorial in causation. Evidence from previous studies^{27,28} suggests that sputum from patients with asthma and products of inflammatory cells such as eosinophil major basic protein possess ciliostatic activity. Environmental factors such as exposure to irritants or chronic infection may cause secondary ultrastructural defects of the cilia that may affect the ciliary beat frequency, as indicated in a number of reports.²⁹ The complex array of inflammatory mediators present in the asthmatic epithelium possesses diverse and opposing effects on ciliary beat frequency.³⁰ In addition, medications may influence ciliary beat frequency. Indeed, both short-acting and long-acting β_2 -agonists increase ciliary beat frequency.³¹ Importantly, in our study, there was no significant correlation between sputum eosinophilia and ciliary beat frequency in subjects with severe asthma, and a significantly reduced ciliary beat frequency was observed in patients with moderate and severe asthma, despite being

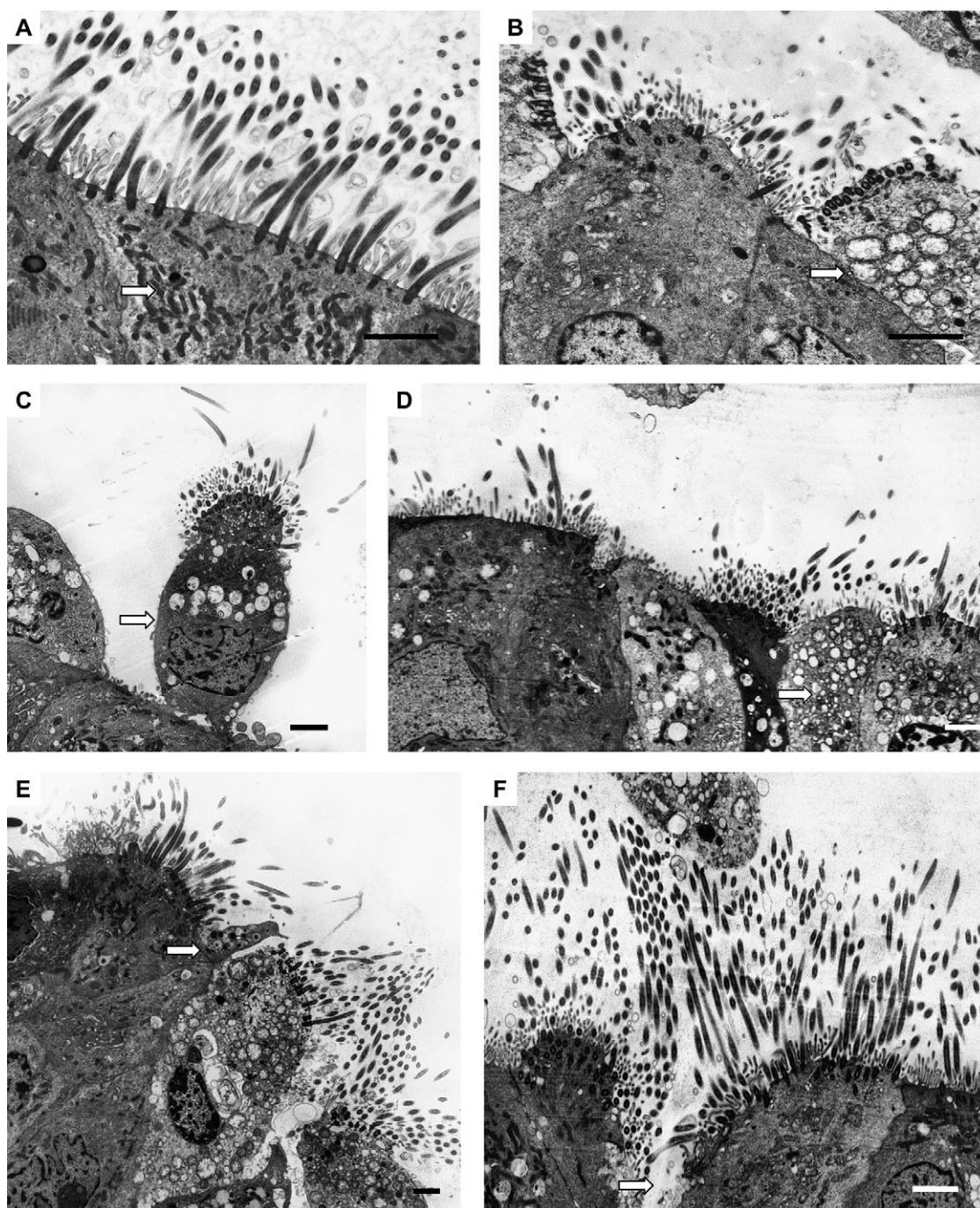


FIG 2. Ciliated respiratory epithelium from a healthy control (**A**) showing normal mitochondria (arrow) and a patient with severe asthma (**B-F**) showing abnormal mitochondria (**B**), projecting cell (**C**), dead cell (**D**), cytoplasmic bleb (**E**), and disruption of tight junction with separation of cells (**F**). Internal scale bar = 2 μ m.

on treatment with long-acting bronchodilators. Therefore, the cause of the dysfunctional ciliated epithelium we observed in severe disease remains to be determined. It should also be noted that although the immotile cilia may be abnormal in that they do not contribute to mucus flow, they may have important mechanosensory and chemosensory functions.³² Of particular interest, we observed significantly increased intracellular ciliary disorientation in the respiratory epithelium of patients with severe asthma compared with healthy controls and patients with mild and moderate

asthma. The magnitude of ciliary disorientation seen in patients with severe asthma is comparable to that previously described as a possible variant of primary ciliary dyskinesia.^{25,33} In primary ciliary dyskinesia, the movement of mucus by cilia is negligible. Although we did not study mucociliary clearance in our subjects and despite the fact that factors such as quantitative and qualitative properties of mucus and airway surface liquid also are known to affect mucociliary clearance, on the basis of available evidence,^{26,34} the degree of ciliary dysfunction that we have

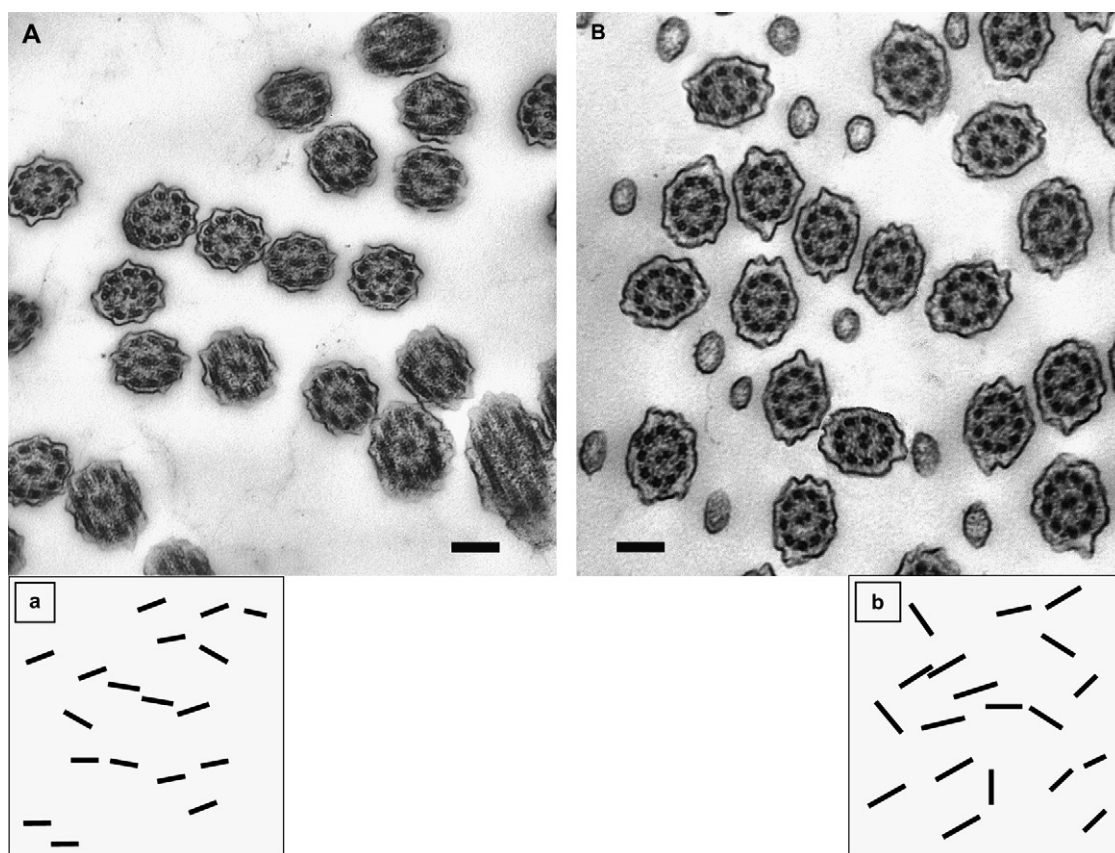


FIG 3. Cross-sectional image of cilia originating from a single ciliated cell of a healthy control (**A**) showing normal ciliary orientation and a patient with severe asthma (**B**) showing ciliary disorientation. *a* and *b*, Distribution of lines drawn parallel to the central pair of microtubules of cilia originating from a single ciliated cell. Internal scale bar = 100 nm.

demonstrated in this study is very likely to correlate with the degree of impairment in mucociliary clearance. If the ciliary abnormalities seen in the patients with severe asthma we studied were present throughout the airways, these patients would have to rely on cough as their main mechanism of mucus clearance. Markedly reduced mucociliary clearance will predispose these patients to secondary bacterial infection, and indeed, asthma has been shown to be an independent risk factor for invasive pneumococcal disease.³⁵ The combination of impaired mucociliary clearance and infection may lead to the development of bronchiectasis. Although asthma and bronchiectasis are two diseases with distinct pathophysiologic processes, their coexistence has been reported in a number of patients, especially in those with severe persistent asthma,³⁶ and despite several caveats, a number of retrospective and cross-sectional cohort studies suggest that asthma may be a predisposing factor in the development of bronchiectasis.^{36,37} In this study, all patients with moderate and severe asthma had high-resolution computed tomography of the chest performed, and 4 (21%) patients with severe asthma had evidence of bronchiectasis.

The ciliary disorientation seen in patients with severe asthma is likely to be a consequence of the chronic inflammation characteristic of asthma or chronic infection³⁸ that may occur in some patients with asthma. Indeed, there is a suggestion that inflammatory mediators such as leukotriene D₄ may impair ciliary orientation.³⁹ In addition, studies done on quail oviduct and *Xenopus*

larval skin suggest that ciliary polarity and orientation are influenced by the normal development of the apical cytoskeleton and normal tissue patterning.^{40,41} Thus, recent evidence of aberrant epithelial repair seen in asthmatic epithelium^{6,7} could also provide a plausible mechanism contributing to ciliary disorientation seen in asthma.

In this study, we also quantified the ultrastructural abnormalities of the respiratory epithelium. Our findings of loss of epithelial integrity and ultrastructural abnormalities are consistent with a number of previous studies showing that asthmatic airway epithelium is structurally abnormal. Evidence supporting epithelial structural damage in asthmatic airways comes largely from earlier studies showing exfoliated epithelial cells in bronchoalveolar lavage samples and marked desquamation, epithelial shedding, and loss of integrity in histopathologic studies on autopsy or endobronchial biopsy specimens.^{42,43} Despite the controversy⁴⁴ regarding the epithelial abnormalities seen in bronchial biopsy studies, accumulating evidence reviewed recently strongly suggests structural abnormalities of the asthmatic epithelium.^{6,7} Emerging evidence also points toward disruption of epithelial tight junctions and other junctional adhesion proteins in asthmatic airways that are responsible for maintaining the normal structural integrity.^{6,7}

One limitation of our study is its cross-sectional design, and therefore the within-subject repeatability of these changes and the response to interventions and exacerbations are uncertain and

need to be further studied. We restricted our study to epithelium derived from central airways, and whether our findings extend into the small airways is unknown. It will be of great interest to investigate the ciliary function and epithelial structure in the peripheral airways of patients with asthma. Whether our findings are specific to asthma or indeed a feature of other airways diseases such as chronic obstructive pulmonary disease or chronic cough needs to be investigated. In spite of these limitations, our measures of ciliary function and epithelial cell morphology are highly repeatable, and therefore the magnitude of ciliary dysfunction in patients with moderate and severe asthma compared with that of controls is striking and is very likely to be clinically important. A further challenge for future studies is to determine whether our findings reflect intrinsic abnormalities of the asthmatic airway epithelium, effects of chronic inflammation, chronic infection, aberrant repair mechanisms, effect of medications, or a combination of these factors.

In summary, our study provides evidence for profound ciliary dysfunction in adults with moderate and severe refractory asthma in stable state, in addition to marked epithelial damage restricted to those with severe disease. The potential direct consequence of this phenotype of secondary ciliary dyskinesia is reduced mucociliary clearance and therefore increased susceptibility to infection, and potentially more prolonged exposure to inhaled particulate pollutants and aeroallergens, all of which have been implicated in the pathophysiology of asthma. These findings extend our current paradigm of severe asthma and present a new therapeutic target.

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Key messages

- Ciliary dysfunction and ultrastructural abnormalities in asthma are related to disease severity.
- Therapy directed at ciliary dysfunction may present a new therapeutic target for asthma.

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VIDEO E1. Sideways view of a ciliated epithelial edge from a healthy control subject showing normal ciliary beat pattern.

VIDEO E2. Sideways view of a ciliated epithelial edge from a patient with severe asthma showing dyskinetic beat pattern.

METHODS

Ciliary beat frequency and beat pattern

Typically, a strip of bronchial epithelium obtained by brushing the bronchus contains a row of adjacent bronchial epithelial cells. Ciliary beat frequency (CBF) was measured and beat pattern assessed on strips of bronchial epithelium using a digital high-speed video system, as described previously with nasal epithelial brushings, within 2 hours of sample collection.^{E1,E2} The high-speed video images were analyzed in a blind fashion. The images were re-analyzed by a second observer (A.R.) and blind on a second occasion by the original observer (B.T.).

The sample was placed in Medium 199 (25 mM hydroxyethyl piperazine ethane sulfonic acid, Earles salt, and L-glutamine [pH 7.3]; Gibco, Leicester, United Kingdom) that contained antibiotic solution (streptomycin 50 $\mu\text{g}\cdot\text{mL}^{-1}$ and penicillin 50 $\mu\text{g}\cdot\text{mL}^{-1}$) and analyzed within 2 hours after collection. The sample was suspended in a chamber created by the separation of a cover slip and a glass slide by 2 adjacent cover slips. The slide was placed on a heated stage (37°C) of a Leitz Diaplan microscope mounted on an antivibration table (Wentworth Laboratories Ltd, Sandy, United Kingdom). Ciliated epithelial strips greater than 50 μm in length and devoid of mucus were observed at 37°C by using a $\times 100$ interference contrast lens. They were recorded by using a digital high-speed video camera (Kodak Ektapro Motion Analyzer; Eastman Kodak, San Diego, Calif) at a frame rate of 400 frames $\cdot\text{s}^{-1}$. Video sequences could be recorded and played back at reduced frame rates or frame by frame. CBF was determined directly from ciliated epithelial strips viewed in a side-ways profile. Groups of beating cilia were identified, and the number of frames required to complete 10 beat cycles was recorded. This was converted to CBF by a simple calculation ($\text{CBF} = [400/\text{number frames for 10 beats}] \times 10$).^{E1} The ciliated epithelial strip ($\geq 50 \mu\text{m}$) projected onto a high-resolution monitor was divided into 10 adjacent areas measuring 5 μm each. One reading of CBF was taken from each area to obtain a total of 10 measurements of CBF along each ciliated epithelial strip. At least 7 epithelial strips, up to a maximum of 10 strips, were analyzed per subject.

The experimental system allowed the ciliary beat pattern to be evaluated in 3 different planes: sideways profile of the ciliated epithelial strip, assessing the ciliated epithelial strip with cilia beating directly toward the observer, and assessing the ciliated epithelial strip from directly above.^{E1,E2} The path taken by a cilium during the beat cycle was analyzed frame by frame. This was characterized and compared with the normal beat pattern^{E1} seen on digital high-speed video analysis. Dyskinesia was defined as an abnormal beat pattern that included reduced beat amplitude, stiff beat pattern, failure to bend along the length of the ciliary shaft, a flickering or twitching motion, and static cilia. The ciliated epithelial strip ($\geq 50 \mu\text{m}$) projected onto a high-resolution monitor was divided into 10 adjacent areas measuring 5 μm each, and the beat pattern of each 5- μm area was assessed to obtain a total of 10 measurements of beat pattern per epithelial strip. The dyskinesia index was calculated as the percentage of dyskinetic cilia within the sample (number of dyskinetic readings/total number of readings for sample $\times 100$). The immotility index^{E3} was calculated as the percentage of immotile cilia within the sample (number of immotile readings/total number of readings for sample $\times 100$).

Transmission electron microscopy

Transmission electron microscopy was performed as described.^{E4} Briefly, on the day of collection, bronchial epithelial samples were fixed in 2.5% glutaraldehyde in Sorenson phosphate buffer. After 48 hours, the sample was postfixed in 1% osmium tetroxide. After rinsing in distilled water, the cells were embedded in a drop of 2% liquid agar at 45°C and allowed to solidify. This bound the cells together during dehydration and ensured that all the strips of epithelium and cilia were randomly oriented. This was processed through to resin by standard transmission electron microscopy techniques. Ultrathin sections were cut at 70 nm. These were collected on 200 mesh thin-bar copper grids and stained in 1% uranyl acetate and counterstained in Reynolds lead phosphate. The sections were then examined by transmission electron microscopy. The grids were analyzed by a grid square search pattern so that all the cells in the sample were analyzed but seen only once. The median (IQR) number of epithelial cells per sample studied was 203.5 (162.8–263.3; range, 106–359). All the cilia that were, by random chance, captured in cross-section adequate for dynein arms to be visualized were assessed.

The ciliated epithelium was assessed, in a blind fashion, for both epithelial and ciliary ultrastructural changes. Epithelial integrity was assessed first by assessing the cell type. The numbers of ciliated cells, unciliated cells, mucus cells, and dead cells were expressed as a percentage of all cells examined. Disruption and damage to the tissue was quantified by using the criteria for epithelial integrity described by Tsang et al.^{E5} Percentages of ciliated cells with loss of cilia, cellular projections (extrusion of cells from the epithelial edge), cytoplasmic blebbing, and mitochondrial damage (manifested as swelling and disruption of mitochondrial cristae) among all cells examined were also calculated. Damage to individual cilia was evaluated by examining ciliary ultrastructure for microtubular and dynein arm defects, and the percentage of cilia with microtubular or dynein arm defects was calculated. Intracellular ciliary orientation, defined as the SD of the angles of lines through the central pair of microtubules of cilia originating from a single ciliated cell, was determined as described previously.^{E6}

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TABLE E1. CBF and beat pattern†

Measurement	Control (n = 9)	Mild asthma (n = 7)	Moderate asthma (n = 7)	Severe asthma (n = 19)	Spearman <i>r</i> (between asthma severity and ciliary function) and <i>P</i> value
CBF (Hz)	10.5 (9.7-11.8)	8.4 (7.5-8.5)	6.5 (4.4-8.5)**	6.7 (6.1-7.6)***	−0.68 (−0.85, −0.44) <i>P</i> < .0001
Dyskinesia index (%)	4 (0-6.7)	13 (9-22)	42 (35-56.1)*	65 (43.1-74.6)***¶	0.86 (0.76, 0.99) <i>P</i> < .0001
Immotility index (%)	0	0	3 (0-4.9)	6.3 (1-9.5)**‡	0.65 (0.59, 0.98) <i>P</i> < .0001

Dyskinesia index, percentage of dyskinetically beating cilia among all cilia examined. Immotility index, percentage of immotile cilia among all cilia examined.

****P* < .001.

***P* < .01.

**P* < .05 compared with control group.

†Data expressed as medians (IQRs).

¶*P* < .01.

‡*P* < .05 compared with mild asthma group.