

Lower airway microbiota associates with inflammatory phenotype in severe preschool wheeze



To the Editor:

Acute wheeze in preschool children accounts for more than 75% of pediatric health care attendances for wheeze/asthma in the United Kingdom.¹ The high morbidity suggests current therapeutic strategies are failing. Preschool wheezers are heterogeneous; many have a disease course clinically distinct from that of childhood type 2-mediated allergic asthma.² However, the pathologic mechanisms underpinning preschool wheeze are poorly understood. Treatment is currently determined by clinical phenotype.¹ Episodic viral wheezers (EVWs) are predominantly prescribed intermittent therapies, and multiple-trigger wheezers (MTWs) are predominantly prescribed maintenance inhaled corticosteroids.¹ However, clinical phenotypes can overlap and change with time.¹ No studies have related lower airway inflammation or infection to clinical wheeze phenotype in children.¹

We hypothesized that lower airway inflammation and microbial dysbiosis during stable disease in preschool children with recurrent severe wheeze (requiring hospitalization or systemic corticosteroids) would be related to clinical wheeze phenotype.¹ We prospectively determined clinical phenotype and analyzed lower airway inflammation and the microbiota in children aged 1 to 6 years with severe wheeze undergoing a clinically indicated bronchoscopy (see Table E1 in this article's Online Repository at www.jacionline.org). Wheeze was doctor diagnosed and confirmed by using a video questionnaire.³ Wheezers were categorized as MTWs or EVWs based on parental report (see the Methods section in this article's Online Repository at www.jacionline.org).² Infection was assessed by using bacterial culture and viral PCR, whereas microbiota profiling was determined by using 16S-based amplicon sequencing. Fiberoptic bronchoscopy and bronchoalveolar lavage (BAL) were performed under general anesthesia.^{4,5} BAL fluid and blood differential leukocyte counts were performed, whole BAL fluid was processed for bacterial culture and viral PCR, and BAL fluid supernatants were processed for microbiota analysis (see the Methods section in this article's Online Repository).⁶ Ethical approval was obtained, and all patients' caregivers provided informed consent.

Thirty-five wheezers (19 male and 16 female patients; median age, 34 months [range, 13.6-70.2 months]) were included

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TABLE I. Patients' demographics and inflammation data

	MTWs (n = 21)	EVW (n = 14)	P value
Male sex, no. (%)	14 (66.7)	5 (35.7)	.09
Age (mo), median (range)	29.2 (13.6-70.2)	33.8 (17.6-57.6)	.90
Weight (kg), median (range)	14.0 (9.2-25.5)	14.0 (10.6-17.6)	.54
Height (cm), median (range)	92.0 (78-116.2)	92.5 (84-115)	.61
Gestational age (wk), median (range)	38 + 5 (35-41)	40 + 0 (36-42)	.06
Birth weight (kg), median (range [n])	3.18 (2.0-3.91 [20])	3.62 (1.41-4.0 [11])	.08
Atopy, no. (%)	6 (28.6)	1 (7.1)	.20
Passive smoking, no. (%)	5 (25.0)	3 (21.4)	.99
Inhaled corticosteroids, no. (%)	12 (57.1)	8 (57.1)	.99
OCS ever, no. (%)	16 (76.2)	9 (64.3)	.47
No. of OCS courses, median (range [n])	1.5 (0-30 [20])	2 (0-16)	.57
Inhaled salbutamol, no. (%)	17 (81.0%)	11 (78.6%)	.99
Oral montelukast, no. (%)	9 (42.9)	7 (50.0)	.74
Oral antibiotics (ever), no. (%)	19 (90.5)	14 (100)	.51
No. of antibiotic courses, median (range)	2 (0-21)	3 (1-12)	.46
Confirmed wheeze, no. (%)	17 (81.0)	8 (57.1)	.27
TRACK score, median (range [n])	50 (10-85 [13])	62.5 (30-75 [6])	.27
PACQLQ score, median (range [n])	4.50 (2.3-7 [16])	5.16 (1.2-7 [8])	.95
Hospitalizations for wheeze, median (range [n])	5 (0-100 [20])	3 (0-16 [13])	.19
Peripheral blood eosinophils (%), median (range [n])	3.4 (0.7-15.5 [20])	5.0 (1.3-14.0)	.48
Peripheral blood neutrophils (%), median (range [n])	39.8 (14.0-68.3 [20])	37.6 (16.0-51.1)	.46
Peripheral blood lymphocytes (%), median (range [n])	44.1 (20.0-67.4 [20])	46.69 (33.3-74.0)	.44
Peripheral blood monocytes (%), median (range [n])	7.1 (4.6-16.9 [20])	6.5 (4.9-11.5)	.57
BAL fluid eosinophils (%), median (range [n])	0.6 (0.0-26.4 [18])	0.9 (0.0-5.7 [13])	.88
BAL fluid neutrophils (%), median (range [n])	7.1 (0.5-67.1 [18])	42.1 (1.4-84.5 [13])	.06
BAL fluid lymphocytes (%), median (range [n])	9.2 (4.4-51.3 [18])	7.4 (0.7-53.4 [13])	.65
BAL macrophages (%), median (range [n])	79.7 (16.5-91.2 [18])	37.8 (9.3-92.8 [13])	.03
Serum IgE (IU/mL), median (range [n])	10.5 (1-2589 [20])	34 (2-495 [13])	.33
Positive bacterial culture, no. (%)	9 (42.9)	7 (50.0)	.79
Positive viral PCR, no. (%)	8 (38.1)	6 (42.9)	.99

Passive smoking is defined by parental report of either parent currently smoking. See the Methods section in this article's Online Repository for details of statistical tests. OCS, Oral corticosteroid; PACQLQ, Paediatric Asthma Caregiver's Quality of Life Questionnaire; TRACK, Test for Respiratory and Asthma Control in Kids.

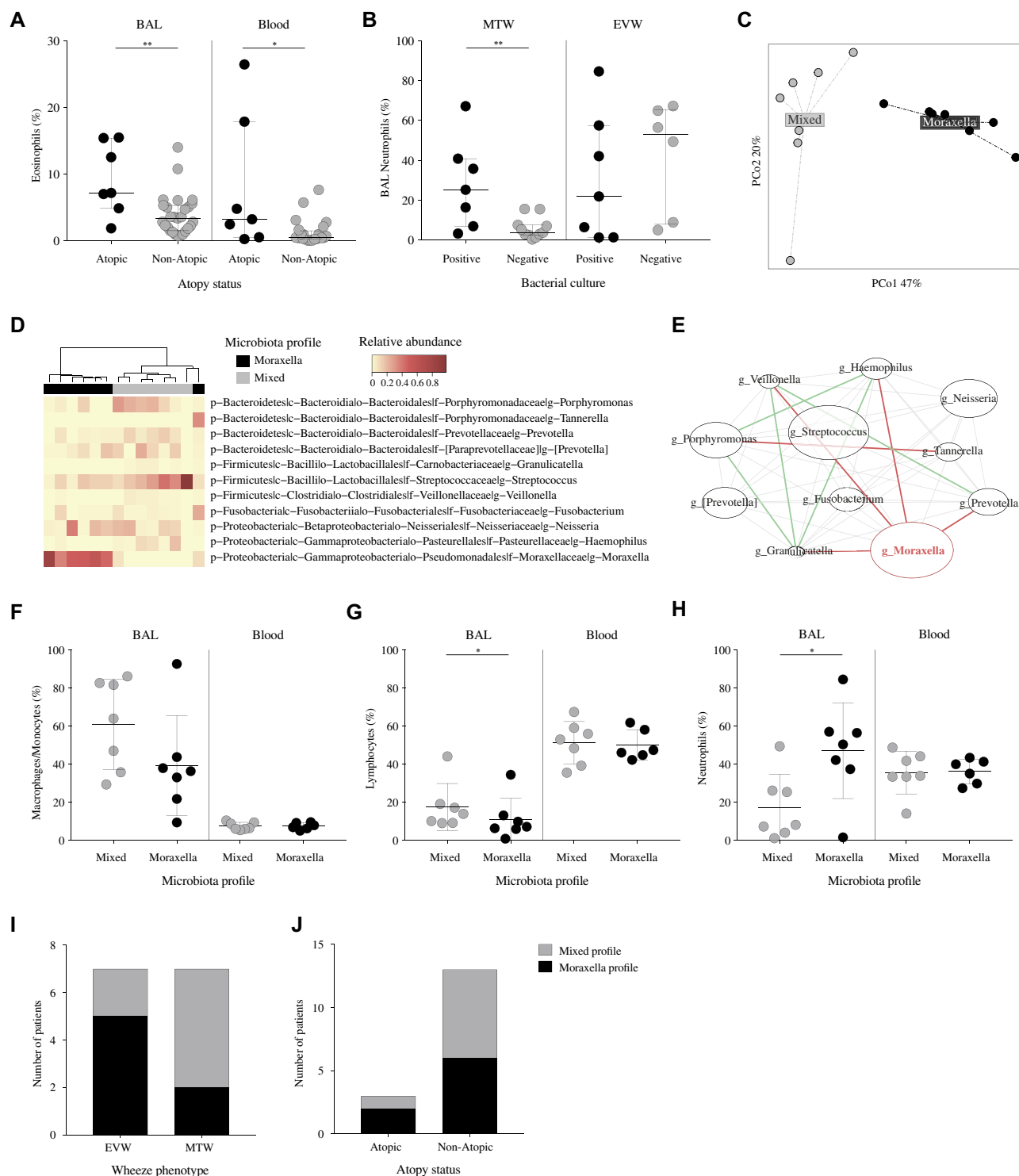


FIG 1. Airway microbial dysbiosis in patients with severe preschool wheeze. **A**, All wheezers' eosinophil percentages. **B**, Neutrophil percentages and bacterial detection in the MTW and EVW groups. **C**, Partitioning around medoids clustering by using principal coordinate analysis. **D** and **E**, Heat map with hierarchical clustering (Fig 1, **D**) and co-occurrence network with positive (green) or negative (red) interactions (Fig 1, **E**). Node sizes are proportional to relative abundance. **F-H**, BAL fluid macrophage, lymphocyte, and neutrophil percentages. **I** and **J**, Microbiota profiles by wheeze phenotype and atopy status. * $P < .05$ and ** $P < .01$.

(Table I). MTWs had more BAL fluid macrophages than EVWs. Although there were no other significant differences in blood or BAL fluid leukocytes between MTWs and EVWs (Table I), EVWs had an approximately 6 times greater median BAL

neutrophil count than MTWs, but there was a wide range within the groups and marked overlap between the groups. The percentage of EVWs and MTWs with atopy and their serum IgE levels were similar (Table I). When all wheezers, regardless of clinical

phenotype, were grouped according to atopic status, blood and BAL fluid eosinophil counts were significantly greater in atopic wheezers compared with those in nonatopic wheezers (Fig 1, A).

Numbers of EVWs and MTWs with positive BAL fluid bacterial culture or viral detection results by using routine clinical analysis were similar (Table I). Sixty percent of all wheezers had either positive bacterial culture or viral detection results, and 26% had both. The most common bacteria cultured were *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, and *Haemophilus influenzae*, and the most common viruses were rhinovirus, bocavirus, and adenovirus.

To examine the relationship between infection and cellular inflammation, we compared BAL fluid granulocytes with culture results and wheeze phenotype. Greater BAL fluid neutrophil counts were associated with positive BAL fluid bacterial cultures in MTWs but not EVWs (Fig 1, B). There was no relationship between BAL fluid neutrophil counts and the presence of a virus or BAL fluid eosinophilia and infection.

Surplus BAL fluid remaining after clinically indicated tests (leukocyte differential, bacterial culture, and viral PCR) was used for microbiome analysis in 26 patients. Six of 26 were insufficient for 16S amplification for sequencing, and a further 6 of 26 were sequenced but were of very low quality, leaving BAL fluid from 14 patients (7 MTWs and 7 EVWs) with high-quality microbiota profiling. Unsupervised analysis of the microbiota in all wheezers revealed 2 distinct profiles referred to hereafter as “mixed” or “*Moraxella*” (Fig 1, C). Indeed, *Moraxella* species was the most discriminant genus between the 2 groups (mean relative abundance, 47.5% compared with 1.2%; Fig 1, D). Interaction networks of the most represented genera (Fig 1, E) revealed 5 significant positive and 5 negative co-occurrences. *Porphyromonas*, *Veillonella*, *Haemophilus*, *Granulicatella*, and *Prevotella* species showed significant co-occurrence. The majority of negative co-occurrences were associated with *Moraxella* species, which is indicative of microbial dysbiosis in its presence.

The *Moraxella* species profile was characterized by lower proportions of BAL fluid macrophages and lymphocytes (Fig 1, F and G) but significantly higher neutrophil counts (Fig 1, H) when compared with the mixed microbiota profile. There were no differences in BAL fluid eosinophil or blood leukocyte counts between the microbiota profiles. Microbiota profiles did not significantly associate with MTW or EVW clinical phenotypes (Fig 1, I) or atopy (Fig 1, J). However, the majority of EVWs had a *Moraxella* profile (Fig 1, I), and EVWs had a trend toward greater BAL neutrophil counts (Table I), suggesting an association between EVW status, microbiota profile, and neutrophilia. BAL fluid microbiota and granulocyte profiles did not correlate with the subject’s age. All wheezers with a *Moraxella* species-predominant microbiota profile also had positive BAL fluid cultures of *M catarrhalis* by using traditional techniques, but 3 of 7 additional wheezers who had not grown *M catarrhalis* were identified by using 16S rDNA gene amplicon sequencing.

To our knowledge, this is the first study investigating lower airway inflammation and microbiota composition in preschool patients with severe wheeze. We found that stratification of patients as EVWs or MTWs did not consistently reflect underlying lower airways inflammatory status or microbiology (viral and bacterial). In contrast, microbiota-based clustering of wheezers correlated with local inflammation. Specifically, the overrepresentation of *Moraxella* taxa and subsequent reduced bacterial diversity reflective of microbiota dysbiosis were linked

with neutrophilia. This is consistent with adult studies reporting lower bacterial diversity and increased prevalence of *Moraxella* species in patients with severe asthma with neutrophilia.^{7,8} By contrast, the mixed microbiota profile was characterized by increased diversity, notably in the abundance of commensal bacterial genera, such as *Streptococcus*, *Prevotella*, *Neisseria*, and *Porphyromonas* species. The latter are known constituents of the healthy adult lung microbiota.

Only a limited number of samples could be analyzed, which is reflective of the difficulty in obtaining these rare lower respiratory tract samples using invasive BAL procedures and the technical considerations surrounding isolating high-quality microbial DNA for sequence analysis. Confirmation in larger numbers is needed, and analysis of mild wheezers would be valuable. Nevertheless, it is striking that even with a modest number, our microbiota and cellular analysis clearly defines 2 distinct patient groups, which was not possible when using standard approaches.

In summary, we have shown there is no consistent relationship between lower airway inflammation or infection and clinical preschool wheeze phenotypes,² suggesting that use of clinical phenotype alone to guide maintenance therapy to prevent wheeze attacks is not appropriate. Assessment of the lower airway microbiota revealed 2 groups; a *Moraxella* species dysbiotic microbiota cluster that associated with airway neutrophilia and a mixed microbiota cluster with a macrophage- and lymphocyte-predominant inflammatory profile. We speculate that antibiotics might be beneficial only for the *Moraxella* species cluster.

We thank Royal Brompton Hospital Consultants Dr Ian Balfour-Lynn, Dr Mark Rosenthal, Dr Claire Hogg, Dr Siobhan Carr, Dr Hui-Leng Tan, and Professor Jane Davies for their help in patient recruitment and sample collection. We thank the parents and children who agreed to take part in the research.

Polly F. M. Robinson, BM, BChir, MRes, MRCPCH^{a,b,*}

Céline Pattaroni, MSc^{c,*}

James Cook, MBBS, MSc, MRCPCH^{a,b}

Lisa Gregory, PhD^a

Aldara Martin Alonso, MRes^a

Louise J. Fleming, MBChB, MD^{a,b}

Clare M. Lloyd, PhD, FRSC^a

Andrew Bush, MD, FRCP, FRCPCH^{a,b}

Benjamin J. Marsland, PhD^{c,*}

Sejal Saglani, BSc(hons), MBChB, MD^{a,b,*}

From ^athe National Heart and Lung Institute, Imperial College, London, United Kingdom; ^bthe Paediatric Respiratory Department, Royal Brompton and Harefield NHS Foundation Trust, London, United Kingdom; and ^cthe Department of Immunology and Pathology, Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, Australia. E-mail: s.saglani@imperial.ac.uk.

*These authors contributed equally to this work.

The project was funded by the British Lung Foundation, UK (grant reference RG14-12). Disclosure of potential conflict of interest: P.F.M. Robinson was a National Institute for Health Research (NIHR) Academic Clinical Fellow and had an NHLI Masters Scholarship. L. J. Fleming is an Asthma UK Senior Clinical Fellow. C. M. Lloyd is a Wellcome Senior Fellow. A. Bush is an NIHR Senior Investigator. S. Saglani is an NIHR Career Development Fellow CDF-2014-07-019. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online December 21, 2018.
https://doi.org/10.1016/j.jaci.2018.12.985

Transcriptome profiling of refractory atopic keratoconjunctivitis by RNA sequencing



To the Editor:

Atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis are severe chronic forms of allergic conjunctivitis that are often accompanied by the formation of giant papillae in the tarsal conjunctivae (see Fig E1 in this article's Online Repository at www.jacionline.org) with prominent T_H2 cytokine profiles.¹ Recent studies have demonstrated the efficacy of tacrolimus for the treatment of AKC/vernal keratoconjunctivitis.² However, there are refractory cases, especially of AKC, despite intense treatments using immune suppressants and glucocorticoids.² To elucidate the pathophysiological characteristics of refractory AKC, we investigated gene expression profiles using tissues of giant papillae obtained from patients with AKC, refractory to tacrolimus treatment for more than 8 weeks (clinical information of the patients is presented in Table E1 in this article's Online Repository at www.jacionline.org). AKC was defined as bilateral chronic inflammation of the conjunctiva and lids associated with atopic dermatitis, often accompanied by corneal pannus formation (arrows, Fig E1) and papillary hypertrophy of the upper tarsal conjunctiva (Fig E1, B, D, and F).¹ It is also associated with superficial keratitis.³ Giant papillae from 3 patients with AKC were obtained during resection surgeries as previously described.⁴ Upper bulbar conjunctivae resected from 3 patients with conjunctivochalasis were used as control samples.⁴ Conjunctivochalasis was defined as a redundant conjunctiva without definite inflammation typically located between the globe

and the lower eyelid. All the procedures were approved by the ethics committees of the Juntendo University School of Medicine and Kyoto Prefectural University School of Medicine. The study was conducted in accordance with the tenets of the Declaration of Helsinki. Total RNA was extracted from the giant papillae and conjunctivochalasis tissues, and RNA sequencing (RNA-seq) was performed with an Illumina HiSeq 1500.

Between 23 and 51 million reads were obtained per sample (36, 24, 36 million for AKC samples and 33, 36, 53 million for conjunctivochalasis samples) by RNA-seq. After trimming and filtering using FastX-Toolkit, 1.9% to 3.2% of the reads were excluded and 94.3% to 96.1% of the remaining reads were mapped to the human genome using HISAT2. Fragments per kilobase of exon per million mapped reads (FPKM) and false discovery rate were calculated using Cufflinks. We defined differentially expressed genes (DEGs) as those with a fold change of greater than 2, FPKM of greater than 10, and false discovery rate of less than 0.05, and found 872 hyperexpressed transcripts in AKC samples and 674 hypoexpressed transcripts in AKC samples (see Fig E2 in this article's Online Repository at www.jacionline.org). We made several lists of DEGs by sorting our results. We first made a DEG list sorted by the fold change of expression intensity (including the DEGs above logfold > 3, which means at least 8-fold changes) with FPKM values greater than 100 in the AKC samples (Table I). One of the findings was the abundance of immunoglobulin-encoded transcripts (among the 872 hyperexpressed DEGs, 47 mapped to immunoglobulin genes). This result was consistent with our previous report showing ectopic lymphoid organs in the giant papillae obtained from patients with AKC,⁴ and a previous report showing immunoglobulin accumulation in giant papillary conjunctivitis.⁵ Taken together, these results suggested that local immunoglobulin production was a feature of chronic allergic conjunctivitis like AKC and giant papillary conjunctivitis. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that the "Staphylococcus aureus infection" pathway was enriched in AKC samples (see Table E2 in this article's Online Repository at www.jacionline.org). Hyperexpressed DEGs included genes within the *S aureus* infection pathway, ICAM1, complement-associated transcripts (C1QC, C1QB, and CFI), and formyl peptide receptors (FPR1 and FPR2), which are known to be associated with process host defense against *S aureus*.⁶ These results seemed to be reasonable considering the fact that microbial infection (especially *S aureus* infection) is one of the clinical features of AKC.³

We also made another DEG list sorted by the order of expression intensity in AKC tissue. Epithelial cell-derived genes, including damage-associated molecular pattern genes (S100A2 and S100A8) and hyperproliferative state-related

TABLE I. List of highly expressed genes in AKC (log2fold > 3, FPKM of AK > 100)

Gene	FPKM (control)	FPKM (AKC)	log2 (fold change)	P value
BPIFA1, SPLUNC-1 antimicrobial protein	0	143.10	Inf	5.00×10^{-05}
CA2, Carbonic anhydrase 2	0.15	134.83	9.86	.0001
LTF, Lactotransferrin	1.14	277.83	7.93	5.00×10^{-05}
POSTN	8.99	2021.92	7.81	5.00×10^{-05}
IGHV3-7	1.97	407.73	7.69	5.00×10^{-05}
CLC, Eosinophil lysophospholipase	1.25	141.87	6.83	5.00×10^{-05}

(Continued)

METHODS

Patient recruitment and study design

Children aged 1 to 6 years undergoing elective clinically indicated bronchoscopy for severe recurrent wheeze were recruited prospectively between September 2015 and August 2016.^{E1} Wheeze was doctor diagnosed and confirmed by using a validated video questionnaire.^{E2} Wheezers were categorized as MTWs or EVWs based on parental report.^{E3} Ethics approval was obtained from the National Research Ethics Committee, Royal Brompton Hospital (RBH) Research and Design Department (15/LO/1885, 08/H0708/3, and 15/SC/0569). All patients' caregivers provided written informed consent. Inclusion and exclusion criteria are detailed in Table E1.

Collection of data on clinical characteristics

Caregivers completed a clinical questionnaire. This included questions on symptom pattern used to phenotype children as either EVWs who wheeze only during acute attacks in association with a clinically diagnosed viral upper respiratory tract infection or MTWs who wheeze both during and between attacks.^{E3} Symptom control was assessed by using the Test for Respiratory and Asthma Control in Kids,^{E4} and symptom effect was assessed by using the Paediatric Caregivers Asthma Quality of Life Questionnaire.^{E5} The questionnaire also included early-life events (gestation, mode of delivery, birth weight, neonatal resuscitation and need for respiratory support, and mode of feeding); family demographics (siblings, atopy, smoking, pets, and urban or rural location); past medical history, including comorbidities and atopy; and current and previous medication use. Retrospective data were collected from electronic medical records at the RBH, where patients were treated. Vaccination status, including for *Haemophilus influenzae B*, was not recorded.

Sample collection and processing

Fiberoptic bronchoscopy was performed after achievement of general anesthesia.^{E6} Three aliquots of 1 mL/kg 0.9% sodium chloride were used for BAL.^{E1} BAL fluid was first processed for clinical indications. BAL cytospin preparations were prepared, and differential leukocyte counts were performed by the RBH laboratory.^{E7} Differential leukocyte counts were performed, as previously described.^{E7} Human tissue was processed in accordance with the Human Tissue Act (2004). Whole BAL fluid was processed at the RBH laboratory for bacterial culture and viral PCR (FTD Respiratory pathogens 21 multiplex kit; Fast Track Diagnostics, Sliema, Malta). Any surplus BAL fluid was stored at -80°C for microbiota analysis. Because BAL fluid was first sent for clinically indicated tests, only BAL fluid that was left over after cytospin had been prepared, and samples sent for bacterial culture and viral PCR could be used for microbiota profiling. To ensure high-quality data, samples that did not meet the quality controls listed below were excluded. Blood was collected for differential leukocyte counts and specific IgE to aeroallergens (house dust mite, cat, dog, and grass pollen). Atopy was defined as more than 1 positive specific IgE level (≥ 0.35 kU/L) or more than 1 positive skin prick test response. Because children were undergoing a clinically indicated procedure, not all recruited patients had blood or BAL fluid samples taken, and thus the sample size for each parameter is stated.

Bacterial DNA isolation and 16S rDNA sequencing

Bacterial DNA was isolated from BAL fluid supernatants, as previously described.^{E8} In addition, DNA extraction and PCR-negative controls were added. Amplification of the 16S rDNA gene was carried out with custom-barcode primers targeting the V1 to V2 region before pooling at equimolar amounts and sequencing on an Illumina MiSeq platform (Illumina, San Diego, Calif), as previously described.^{E9}

16S rDNA sequence analysis

Sequences were quality filtered (quality Phred score $Q < 20$, >3 low-quality base calls, and $>75\%$ of their original length), and chimeras were removed by using usearch61, clustered into operational taxonomic units, and

assigned taxonomy by using Quantitative Insights into Microbial Ecology (QIIME, version 1.9)^{E10} software with usearch61 with the Greengenes (version 13.5) database.^{E11} Diversity was estimated with the Shannon index, as calculated in QIIME. All downstream analyses were performed with R statistical software by using an operational taxonomic unit table rarefied at a sequencing depth of 50,000 reads. Samples with less than 5,000 quality reads or clustering with extraction or PCR-negative controls were removed from the data set. β -Diversity was estimated by using principal coordinate analysis on the Bray-Curtis dissimilarity matrix at the genus level. Samples were clustered by using the partitioning around medoids algorithm. A heat map showing relative abundance of the most abundant genera ($>1\%$ relative abundance) and a dendrogram were drawn by using Ward hierarchical clustering (Bray-Curtis distance, genus level). A co-occurrence network was built with the Sparse Correlations for Compositional (SparCC) data algorithm^{E12} on the most abundant genera ($>1\%$ relative abundance). Significant associations were defined as positive SparCC data correlations with a P value of less than .05 (pseudo- P values were calculated by using a bootstrap procedure with 999 random permutations and 999 iterations for each SparCC data calculation). Network visualization (undirected positive or negative co-occurrence) was carried out with the Fruchterman-Reingold layout algorithm and the igraph package.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (version 7; GraphPad Software, La Jolla, Calif). A power calculation was unable to be performed because of insufficient data to inform it. Distribution of data was assessed by using the D'Agostino-Pearson test. For normally distributed continuous data, differences between groups were assessed by using the Student t test. For nonparametric data, intergroup differences were analyzed by using the Mann-Whitney test. Categorical data were analyzed by using the 2-tailed Fisher exact test. Values are expressed as medians with interquartile ranges. Statistical significance was accepted at P values of less than .05 and less than .01. For histologic counts, intraobserver variability was calculated by analyzing a subset of samples 3 times with a minimum of 2 weeks between counts and presented as the coefficient of variation. A subset of samples was counted by a minimum of 2 investigators, and interobserver variability was analyzed with Bland-Altman plots for 2 observers or as the coefficient of variation for 3 observers. Because participants were undergoing clinically indicated tests, not all patients had blood and BAL fluid samples taken. Additionally, a subset of samples did not meet the RBH laboratory's quality control standards and were excluded. Hence the sample size for each parameter is stated in the table or figure legend.

Extended Fig 1 legend

Fig 1. Airway microbial dysbiosis in patients with severe preschool wheeze. **A**, Eosinophil percentage according to atopic status in all wheezers in peripheral blood (data available for atopic [$n = 7$; black circles] and non-atopic [$n = 27$; gray circles] subjects) and BAL fluid (data available for atopic [$n = 7$] and nonatopic [$n = 24$] subjects). Each point represents an individual child. **B**, For microbiology in MTWs, BAL fluid neutrophil percentages related to BAL fluid positive for bacterial culture. Black circles represent positive microbiological results, and gray circles represent negative microbiological results (data are available for positive [$n = 7$] and negative [$n = 11$] results). For microbiology in EVWs, data are available for positive ($n = 7$) and negative ($n = 6$) results. Each point represents an individual child. **C**, Principal coordinate analysis score plot on Bray-Curtis distance at the genus level, with each dot representing an individual patient. Colors indicate differential mixed (gray) and *Moraxella* (black) microbiota profiles, as determined by using an unbiased partitioning around medoids clustering algorithm, with the rectangle showing the cluster centroid. **D**, Heat map of the most abundant genera ($>1\%$ mean relative abundance), with each column representing an individual patient. The color-scale bar indicates operational taxonomic units (OTU) relative abundance from 0% to 100%. The dendrogram represents Ward hierarchical clustering on Bray-Curtis distance at the genus level. **E**, Taxonomic co-occurrence network of the most abundant genera

(>1% mean relative abundance) on the total data set using the SparCC data algorithm. *Thick edges* indicate the extent to which bacterial genera positively (green) or negatively (red) co-occur, and node size is proportional to genus mean relative abundance. Only significant correlations (1-sided $P < .05$) are represented in color. **F-H**, Results of cytospin cell differentials showing percentages of macrophages/monocytes, lymphocytes, and neutrophils in BAL fluid and peripheral blood in relation to microbiota profiles. Each *point* represents an individual child. **I and J**, Distribution of microbiota profiles (gray, mixed profile; black, *Moraxella* profile) by clinical wheeze phenotype (Fig 1, I) and atopy status (Fig 1, J). Statistics represent results of the D'Agostino and Pearson normality test, followed by the Mann-Whitney test for nonparametric data (Fig 1, A, B, and F-H). * $P < .05$ and ** $P < .01$.

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TABLE E1. Inclusion and exclusion criteria for patient recruitment

Inclusion criteria	Exclusion criteria
Age 12-72 mo	Known diagnosis of chronic lung disease
Undergoing elective and clinically indicated bronchoscopy between September 2015 and August 2016	Cystic fibrosis
Gestation of 36 wk or greater at birth	Primary ciliary dyskinesia
Recurrent episodes of wheeze and difficulty breathing severe enough to merit referral to a pediatric pulmonologist	Interstitial lung disease
	Dysfunctional breathing
	Psychosocial problems
Parent/guardian able to provide written informed consent	Panic attacks
Able to give age-appropriate assent	Acute illness or recent exacerbation