

Assessing the unified airway hypothesis in children via transcriptional profiling of the airway epithelium

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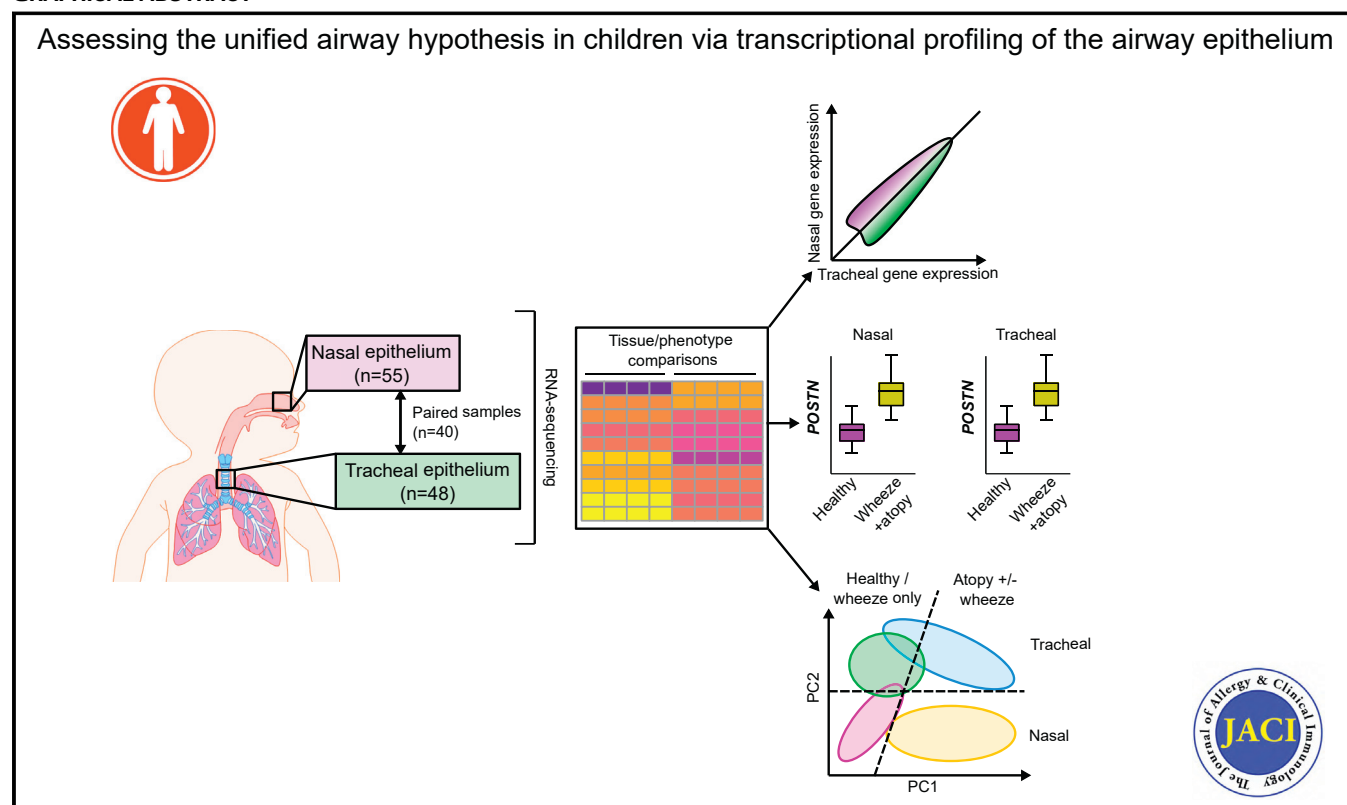
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GRAPHICAL ABSTRACT



Background: Emerging evidence suggests that disease vulnerability is expressed throughout the airways, the so-called unified airway hypothesis, but the evidence to support this is predominantly indirect.

Objectives: We sought to establish the transcriptomic profiles of the upper and lower airways and determine their level of similarity irrespective of airway symptoms (wheeze) and allergy.

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Methods: We performed RNA sequencing on upper and lower airway epithelial cells from 63 children with or without wheeze and accompanying atopy, using differential gene expression and gene coexpression analyses to determine transcriptional similarity.

Results: We observed approximately 91% homology in the expressed genes between the 2 sites. When coexpressed genes were grouped into modules relating to biological functions, all were found to be conserved between the 2 regions, resulting in a consensus network containing 16 modules associated with ribosomal function, metabolism, gene expression, mitochondrial activity, and antiviral responses through IFN activity. Although symptom-associated gene expression changes were more prominent in the lower airway, they were reflected in nasal epithelium and included IL-1 receptor like 1, prostaglandin-endoperoxide synthase 1, *CCL26*, and periostin. Through network analysis we identified a cluster of coexpressed genes associated with atopic wheeze in the lower airway, which could equally distinguish atopic and nonatopic phenotypes in upper airway samples.

Conclusions: We show that the upper and lower airways are significantly conserved in their transcriptional composition, and that variations associated with disease are present in both nasal and tracheal epithelium. Findings from this study supporting a unified airway imply that clinical insight regarding the lower airway in health and disease can be gained from studying the nasal epithelium. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: Airway epithelium, unified airway hypothesis, transcriptomics, gene expression, biological processes

The “unified airway hypothesis” postulates that diseases of the upper and lower airways are both manifestations of a single process.¹ Its principle lies in the concept of a continuous respiratory tract lined with respiratory epithelial cells encompassing the nose and middle ear, which extends to the terminal bronchioles.² Thus, under disease settings, a pathological process in one region of the airway would affect the function of the entire airway.³ This is particularly evident in chronic airway diseases such as chronic rhinosinusitis and asthma. These diseases have traditionally been evaluated and treated as separate disorders, but advances in the understanding and knowledge of the underlying processes have moved opinion toward the concept of unifying their management.⁴

The upper and lower respiratory tracts share many anatomical and histological properties, as well as the airflow into and out of the lungs.⁵ They also share a common susceptibility and response to different allergens, infections, and pollutants.⁶⁻⁹ In the setting of asthma, comparable inflammatory cell profiles, particularly in T cells, mast cells, and macrophages, have been reported for both the upper and lower respiratory tracts.^{10,11} In addition, similar chemokine and cytokine production has also been reported including IL-5, IL-4, IL-13, chemokine (C-C motif) ligand (CCL)5, and GM-CSF levels.^{12,13} In contrast, eosinophil infiltration, epithelial shedding, and basement membrane thickness have all been reported to be greater in the lower airway of patients with asthma.^{10,14} Furthermore, the highly developed vasculature evident in the nose contrasts with the smooth muscle bundles that surround the tracheal/bronchial airways.¹⁵ This dichotomy highlights the need for additional clarification as to whether there

Abbreviations used

CCL:	Chemokine (C-C motif) ligand
CDH:	Cadherin
CLCA1:	Chloride channel accessory 1
CST1:	Cystatin 1
CST2:	Cystatin 2
CST4:	Cystatin 4
IL1RL1:	IL-1 receptor like 1
KRT:	Keratin
NTRK1:	Neurotrophic receptor tyrosine kinase 1
POSTN:	Periostin

exists a unified airway, especially because the complex interactions between the nose and the lung are not well understood but may have important implications for the pathogenesis of respiratory diseases.

In this study, we implemented a transcriptional profiling–based approach to systematically analyze 63 children with or without atopy and/or wheeze to explore similarities and differences in gene expression between the upper and lower respiratory tracts. Specifically, we tested the hypothesis that the transcriptomic profiles of both the nasal and lower tracheal epithelium would be comparable. Using weighted correlation network analysis on all study participants, we identified 16 modules of coexpressed (and functionally enriched) genes to be conserved between the lower and upper airways. Furthermore, we identified an atopic-wheeze gene signature within tracheal epithelium that was equally able to distinguish between atopic and nonatopic phenotypes within nasal epithelium. These findings provide insight into the intrinsic biological differences inherent to epithelium in the context of airway symptoms and support the concept of a unified airway. Our data suggest that overall, there is conservation of the underlying transcriptional networks between the lower and upper airway epithelium in terms of the biological processes that are active at each site; however, some regional specificity in terms of relative gene expression levels is evident.

METHODS

Study population

The study was approved by the Perth Children’s Hospital and St John of God Hospital’s human ethics committees, and written consent was obtained from each participant’s legal guardian after being fully informed about the purpose of the study. Airway samples were obtained from 63 children undergoing elective surgery for non–respiratory-related conditions (Table 1) and those symptomatic for an existing bacterial or viral chest infection were excluded from the study. Children were identified as having a history of wheeze based on reported chest wheezing on auscultation by each participant’s treating physician. All had regular documented episodes of recurrent and persistent wheeze in the preceding year, complemented with a history of documented episodes more than 12 months before recruitment. A smaller number of older children with recurrent wheeze were also diagnosed with asthma, which was defined as physician-diagnosed on the basis of physician-documented wheezing episodes in the 12 months preceding their recruitment and confirmed by positive responses on the International Study of Asthma and Allergies in Children and American Thoracic Society respiratory questionnaires.^{16,17} Atopy was determined by a positive radioallergen sorbent test result to a designated panel of allergens (house-dust mite, mixed grasses, various animal hair, and different molds). Children were stratified into 4 groups for subanalysis: (1) healthy, (2) atopy no

TABLE I. Demographic information for study participants

Information	Healthy controls	Wheeze with atopy	Atopy only	Wheeze only	P value
No. of participants	16	15	16	16	
Samples from participants:					
Paired TEC and NEC	13	10	13	4	
TEC only	2	1	2	3	
NEC only	1	4	1	9	
Age (y), median (range)	3.3 (2.8-6.8)	6.4 (4.0-18.3)	3.8 (2.8-5.5)	3.5 (2.8-7.6)	.052
Sex: male (%)	50	60	50	56	.81
RAST results (% positive):					
Grass pollen	0	80	38	0	<.0001
Animal	0	60	38	0	<.0001
Mold	0	40	25	0	.0035
Egg white	0	33	44	0	.0012
House-dust mite	0	53	38	0	.0002
Past wheeze (%)	19	93	31	100	<.0001
Current wheeze (%)	19	80	19	75	.0005
Hay fever (%)	6	87	44	31	<.0001
Family history of hay fever (%)	75	87	75	75	.83
Eczema (%)	19	60	75	37	.0084
Family history of eczema (%)	37	53	50	75	.19
Parental asthma (%)	37	*40	37	87	.01
Parental smoking (%)	19	13	6	44	.051

NEC, Nasal epithelial cell; TEC, tracheal epithelial cell.

For comparison of age across groups, the *P* value was derived from Kruskal-Wallis test with Dunn's multiple comparisons test for nonparametric data (data for each group checked using the D'Agostino and Pearson normality test). Age is reported as median (interquartile range). For comparisons of categorical variables, *P* values were derived from a χ^2 test across the 4 groups. All percentages are rounded to the nearest whole number.

*Data were missing for 1 participant.

wheeze (atopy alone), (3) wheeze no atopy (wheeze alone), and (4) wheeze and atopy.

Sampling procedure and cell types

Lower airway epithelial cells (AECs) were harvested via transalaryngeal, nonbronchoscopic brushing of the tracheal mucosa of children via an endotracheal tube as previously described.¹⁸⁻²⁰ Matched upper AECs were also obtained and were executed using a rotational movement of the brush against the nasal turbinate. The brush tip was then inserted and cut off into cold sterile media (RPMI-1640) containing 20% heat-inactivated FCS. The process was repeated at least once more before the samples were transported back to the laboratory on ice for immediate processing. An unsheathed soft nylon cytology brush (BC 25105; Shinjuku, Tokyo, Japan) was used to sample both regions and performed by the same physician for all participants to minimize sample variance and potential study confounders.

RNA isolation and sequencing

Collection tubes, containing nasal or tracheal AECs, were vortexed for a period to disrupt the cells off the brush and the larger cell clumps. The brushes are removed and placed into another collection tube that was then vortexed for a period to release any remaining cells. The collection media was then pooled, centrifuged at 500g for 7 minutes at 4°C, and resuspended in 5 mL of collection media. A cell count was then performed and approximately 1 million AECs were fractionated out, pelleted, and resuspended in 350 μ L RLT buffer containing 1% (v/v) β -mercaptoethanol (QIAGEN, Hilden, Germany) and stored at -80°C until all samples were obtained. RNA was extracted using the Ambion Purelink RNA mini kit (Thermo Scientific, Waltham, Mass) per the manufacturer's instructions and appropriate RNA samples shipped to the Australian Genome Research Facility for library preparation (TruSeq Stranded mRNA Library Prep Kit; Illumina, San Diego, Calif) and sequencing (Illumina HiSeq2500, 50-bp single-end reads, v4 chemistry). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif); median RNA integrity number was 8.3 (range, 7.8-9). The RNA-sequencing data have been deposited in the National

Center for Biotechnology Information's Gene Expression Omnibus,²¹ and are accessible through GEO Series accession number GSE118761 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118761>).

Data analysis

Raw RNA-sequencing data were quality assessed using FastQC²² (v0.11.3) and aligned to the human genome (hg19) using HISAT2²³ (v2.0.4). Four samples from different individuals (2 nasal, 2 tracheal) failed quality control checks, and were excluded from downstream analyses. Gene-level quantitation of aligned reads was performed using the summarizeOverlaps() function from the Bioconductor GenomicAlignments package,²⁴ and postalignment quality control using SAMStat²⁵ (v1.5.2). Differentially expressed genes were identified between tracheal and nasal epithelial samples using the Bioconductor package limma-voom,²⁶ with paired samples taken into account through the duplicateCorrelation() function. Differentially expressed genes were identified between study groups using the Bioconductor package DESeq2,²⁷ with model adjustments for age and sex. *P* values for both analyses were adjusted for multiple testing using the Benjamini-Hochberg method, and those less than .05 were considered significant. The weighted gene coexpression network analysis²⁸ algorithm was used to construct gene networks for nasal and tracheal epithelial cells separately, on the basis of gene expression from all study participants. Biological characterization of the differentially expressed genes or consensus network modules was performed using InnateDB²⁹ to identify significantly overrepresented pathways. For comparison to the skin data set, raw fastq sequencing files were downloaded from the Gene Expression Omnibus²¹ (GSE54456) and processed through the same pipeline as our airway epithelial samples, for optimal comparison between tissues. The skin data set was filtered and normalized using identical methods as for the airway epithelial samples, as described in text.

Statistical methods

Statistical analyses of demographic data were performed using Prism (v7; GraphPad Software, San Diego, Calif). We used the Kruskal-Wallis test with Dunn's multiple comparisons test for continuous variables (data for each group

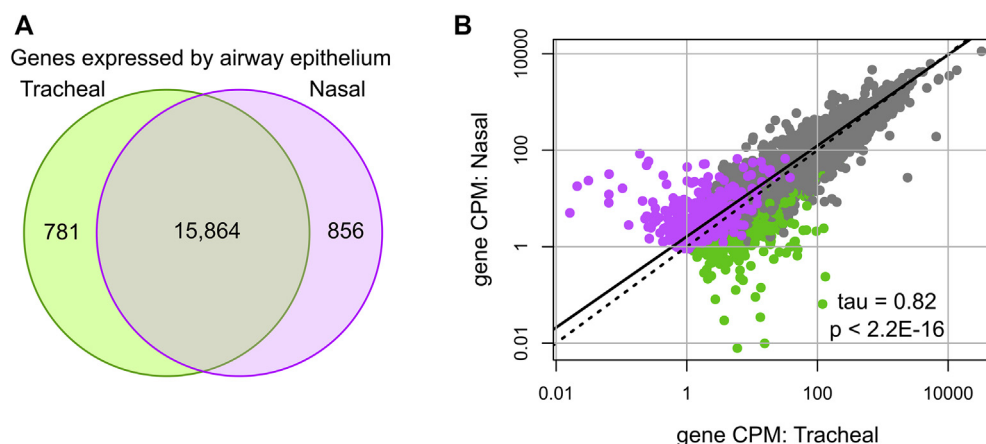


FIG 1. Conserved transcriptional profiles between tracheal and nasal epithelium. **A**, Venn diagram comparing genes with evidence of expression in all tracheal, and all nasal epithelial, samples independent of asthma or atopy. **B**, Scatter plot of the average gene counts per million (CPM) reads, for all expressed genes between tracheal and nasal epithelial samples (across all groups). Colors represent genes identified as more frequently expressed in tracheal or nasal epithelium (green and purple, respectively), or common to both sites (gray).

were checked using the D'Agostino and Pearson normality test) and chi-square tests for categorical variables. All other statistics obtained outside of InnateDB or Ingenuity Pathway Analysis were performed in the R environment (v3.3.3). Correlation of average gene expression between tracheal and nasal epithelium was done using the Kendall rank correlation coefficient for nonparametric data. The modulePreservation() function (which calculates pair-wise module preservation statistics) was used to compare individual coexpression networks for tracheal and nasal epithelium, where results are summarized into a Z score of overall module preservation between the networks.

RESULTS

Comparison of gene expression profiles between tracheal and nasal epithelium, independent of wheeze or atopy

Demographic data for all study participants are presented in Table I, and a summary of all analyses performed is presented in Figure E1 in this article's Online Repository at www.jacionline.org. RNA sequencing resulted in an average of 20 million mapped reads per sample (range, 13.7-25 million). To initially assess the composition of tracheal and nasal epithelium across all samples, we compared the overlap of expressed genes between the 2 sites, using a count per million threshold corresponding to a count of at least 10 in all nasal or tracheal samples. This is a standard filtering strategy for removing low or nonexpressed genes.³⁰ We identified 16,645 and 16,720 genes expressed in tracheal and nasal epithelium, respectively (independent of wheeze or atopy), of which 15,864 (~91%) were expressed in both sites, whereas the remainder were more frequently expressed in either site (Fig 1, A). Overall, gene expression was highly correlated between tracheal and nasal epithelium (Kendall's tau = 0.82; $P < 2.2 \times 10^{-16}$) (Fig 1, B). Next, to confirm whether our gene expression profiles were specific to the airway, or common to other types of epithelium, we compared our data set with that derived from healthy skin punch biopsies³¹ ($n = 82$). Using the same filtering strategy as for the airway samples, we identified 15,784 genes expressed in the skin epithelium, of which 74% to 75% were also expressed in the airway epithelium (see Fig E2 in this article's Online Repository at www.jacionline.org). In general, weaker correlations

were observed between gene expression profiles from the skin and each airway tissue (Kendall's tau = 0.46 and 0.50 for correlations with tracheal and nasal samples, respectively; Fig E2). In addition, to more thoroughly investigate the differences between airway sites, we tested for differentially expressed genes between tracheal and nasal epithelial samples independent of wheeze/atopy, and identified 2467 genes with an absolute fold-change difference in expression of more than 2 between tissues (see Table E1 in this article's Online Repository at www.jacionline.org). We then ranked these genes by highest absolute fold-change, to characterize the most divergent genes. Genes with the greatest dichotomy in expression that were most highly expressed in tracheal epithelium included *MARCO* (involved in innate immune responses), *FABP4* (functions in lung inflammation/angiogenesis), and the surfactant protein-coding genes *SFTA3* and *NKX2-1* (transcription factor involved in lung development). Those most highly expressed in nasal epithelium included a range of transcription factors (*FOXG1*, *PAX7*, *OTX2*, *SIX3*, *SP8*, *PAX3*) alongside the keratin-encoding gene *KRT24*.

We next constructed weighted coexpression gene networks for tracheal and nasal epithelium in parallel (across all samples), as a holistic approach to understanding the functional organization of the transcriptome at each site.³² This analysis works to identify clusters (or modules) of genes with similar patterns of gene expression across each tissue, and provides biological insight on the basis of the principal that genes with highly correlated patterns of expression are likely to participate in the same biological process. Here, we identified 17 network modules within tracheal epithelium, and 8 modules within the nasal epithelium. We next identified significantly overrepresented biological pathways and gene ontology terms for each module in the tracheal and nasal network (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org). A comparison of these 2 site-specific networks revealed a significant overlap in structure; that is, each nasal network module corresponded to 1 or more tracheal network modules (Fig 2, A). This was confirmed through additional module preservation statistics that test how well the modules from one network are preserved in another. Each network module from the nasal network was significantly preserved in

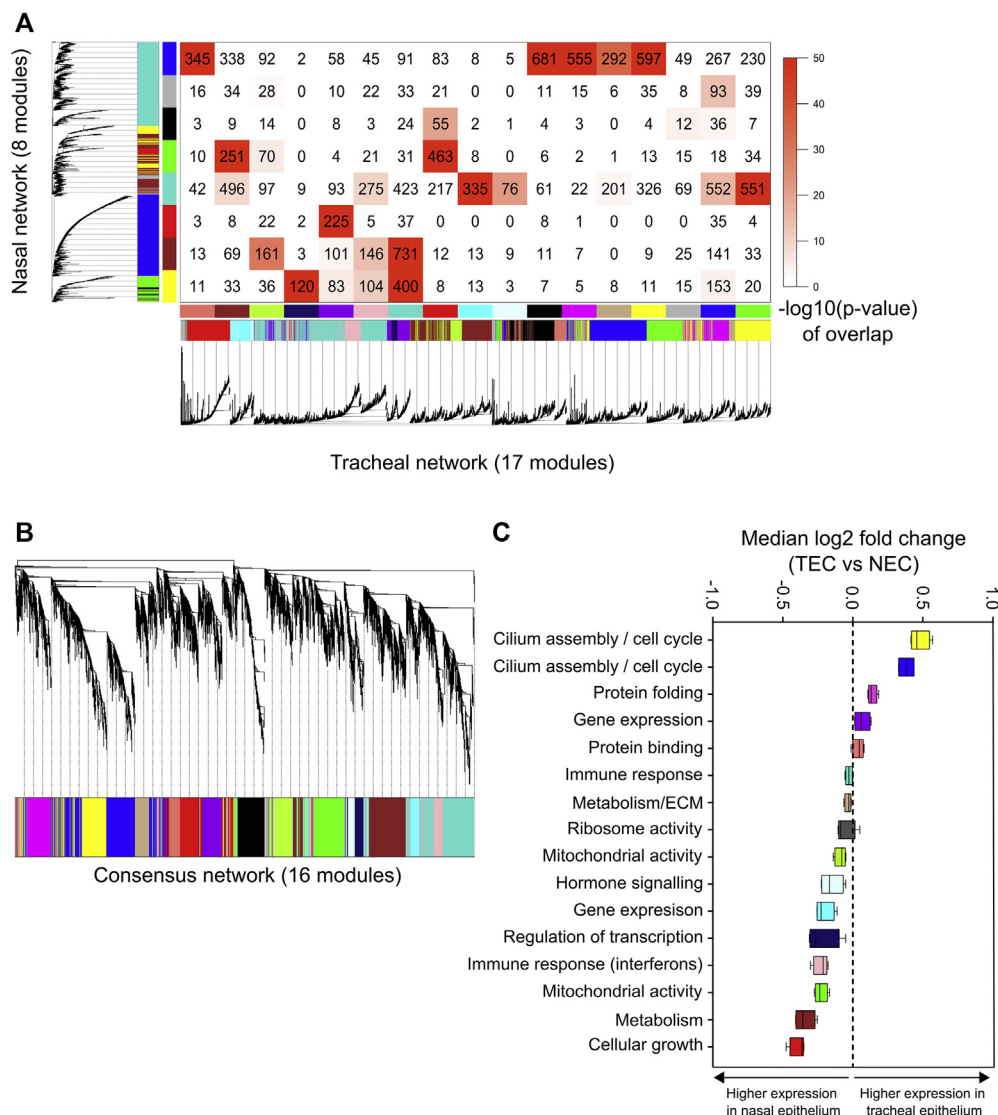


FIG 2. Identification of a consensus gene expression network between tracheal and nasal epithelium. **A**, Weighted gene coexpression network analysis was used to identify modules of coexpressed genes within the upper or lower airway. Cluster dendrograms for the tracheal and nasal networks containing 17 and 8 modules, respectively, are shown alongside the correspondence matrix between the 2 networks. Here, numbers within the table represent the number of genes that overlap between the modules being compared, where a stronger color indicates a more significant overlap based on a Fisher exact test *P* value. **B**, The consensus network containing 16 modules that are shared across the tracheal and nasal networks. Each network module is represented by a different color. **C**, The relative expression of all genes within each consensus module represented as the median log₂ fold-change between tracheal and nasal epithelial cell samples, where box plots represent the results across all 4 study groups; that is, comparisons between tissues were performed independently for each phenotype based on atopy/wheeze. *ECM*, Extra cellular matrix.

the tracheal network (Table II) and vice versa (Table III); a *Z* score of more than 10 indicates strong evidence of module preservation, and the average across networks was 36.8 (range, 14–67). Given the similarities between the tracheal and nasal networks, we next built a consensus network, where the resulting network modules represent robust gene coexpression patterns common to both tracheal and nasal epithelium. The consensus network contained 16 modules of coexpressed genes (Fig 2, B), and each module of the consensus network significantly overlapped with at least 1 module from both tracheal and nasal epithelial networks (see Fig E3 in this article's Online Repository at www.jacionline.org).

Most of the consensus network modules were associated with generalized cellular processes (ribosomal function, metabolism, gene expression, mitochondrial activity), whereas 2 modules were associated with immune responses, one being specific for IFN-mediated antiviral responses (see Table E4 in this article's Online Repository at www.jacionline.org). To assess the relative expression of each consensus network module in each tissue and within each study group, the median fold-change difference in expression of the consensus module genes was measured between tracheal and nasal samples, and was less than 1 across all phenotypes (Fig 2, C). This suggests a modest

TABLE II. Nasal epithelial cell network module preservation within tracheal epithelial cell network modules

Nasal network module		Generalized biological function (Table E1)	Module size	Preservation Z-score summary
1	Brown	Immune response, antigen presentation	1484	57.8
2	Green	Ribosome activity	947	56.6
3	Red	Innate immune response, IFN signaling	350	56.5
4	Blue	Cell cycle, centrosome activity	3738	49.1
5	Yellow	Adaptive immune response, B- and T-cell activity	1030	38.5
6	Turquoise	Metabolism, growth factor signaling	3845	15.6
7	Black	Cell cycle	185	15.2
8	Gray	No collective function (contains genes not associated with any module)	371	2.3
Total genes in network				11,950

TABLE III. Tracheal epithelial cell network module preservation within nasal epithelial cell network modules

Tracheal network module		Generalized biological function (Table E2)	Module size	Preservation Z-score summary
1	Cyan	Regulation of transcription	379	67.0
2	Black	Cilium/cell cycle	789	61.3
3	Magenta	Cilium activity and hedgehog signaling	610	58.6
4	Red	Ribosome activity	859	46.3
5	Turquoise	Immune response	1770	45.4
6	Midnight blue	Immune response (adaptive)	136	39.1
7	Salmon	Cell cycle/movement	443	34.1
8	Light cyan	Apoptosis	94	30.7
9	Purple	Antiviral immune response (IFNs)	582	30.4
10	Tan	NOTCH signaling/metabolism	508	27.9
11	Brown	Mitochondrial activity	1238	27.5
12	Yellow	Transcription, gene expression	995	24.1
13	Green yellow	Gene expression, proteasome activity	520	21.1
14	Blue	Regulation of gene expression (chromatin activity)	1295	14.8
15	Pink	Lysosome, phagosome activity	621	14.4
16	Green	Cellular growth	918	13.9
17	Gray	No collective function (contains genes not associated with any module)	193	6.6
Total genes in network				11,950

TABLE IV. Summary of differentially expressed genes between atopic and/or asthmatic patients and nonatopic healthy controls in tracheal and nasal epithelium

Contrast: description	No. of differentially expressed genes (adjusted <i>P</i> value <.05)		
	Tracheal	Nasal	Overlap
Wheeze with atopy vs healthy controls: Effect of wheeze and asthma	340 (98 down, 242 up)	103 (13 down, 90 up)	49 (2 down, 47 up)
Atopy only vs healthy controls: Effect of atopy only	95 (55 down, 40 up)	38 (1 down, 37 up)	18 (1 down, 17 up)
Wheeze only vs healthy controls: Effect of wheeze only	8 (3 down, 5 up)	41 (25 down, 16 up)	4 (1 down, 3 up)
Wheeze with atopy vs atopy only: Effect of wheeze in presence of atopy	34 (23 down, 11 up)	3 (1 down, 2 up)	2 (1 down, 1 up)

difference in the relative expression of consensus modules between tissues, independent of atopy/wheeze (Fig E4 in this article's Online Repository at www.jacionline.org presents the results for each study group individually). Of note, modules associated with cilium assembly and cell cycle activity (yellow and blue) were more highly expressed in the tracheal epithelium, whereas nasal epithelium was associated with increased expression of modules involving growth and metabolism (red and brown). Taken together, these results demonstrate overall conservation of the underlying transcriptional networks between tracheal and nasal epithelium, while specific gene expression differences relate to site-specific functions.

Gene expression changes associated with wheeze and atopy occur in both nasal and tracheal epithelium

To identify gene expression changes associated with atopy and/or wheeze within the epithelium, differential gene expression analyses were performed contrasting each group against healthy controls. Specifically, these comparisons inform on the gene expression changes related to the combined effect of wheeze and atopy (wheeze with atopy vs healthy controls), atopy only (atopy vs healthy controls), or wheeze only (wheeze vs healthy controls). As an alternative approach to identifying gene signatures attributed to wheeze only, we also compared atopics with or

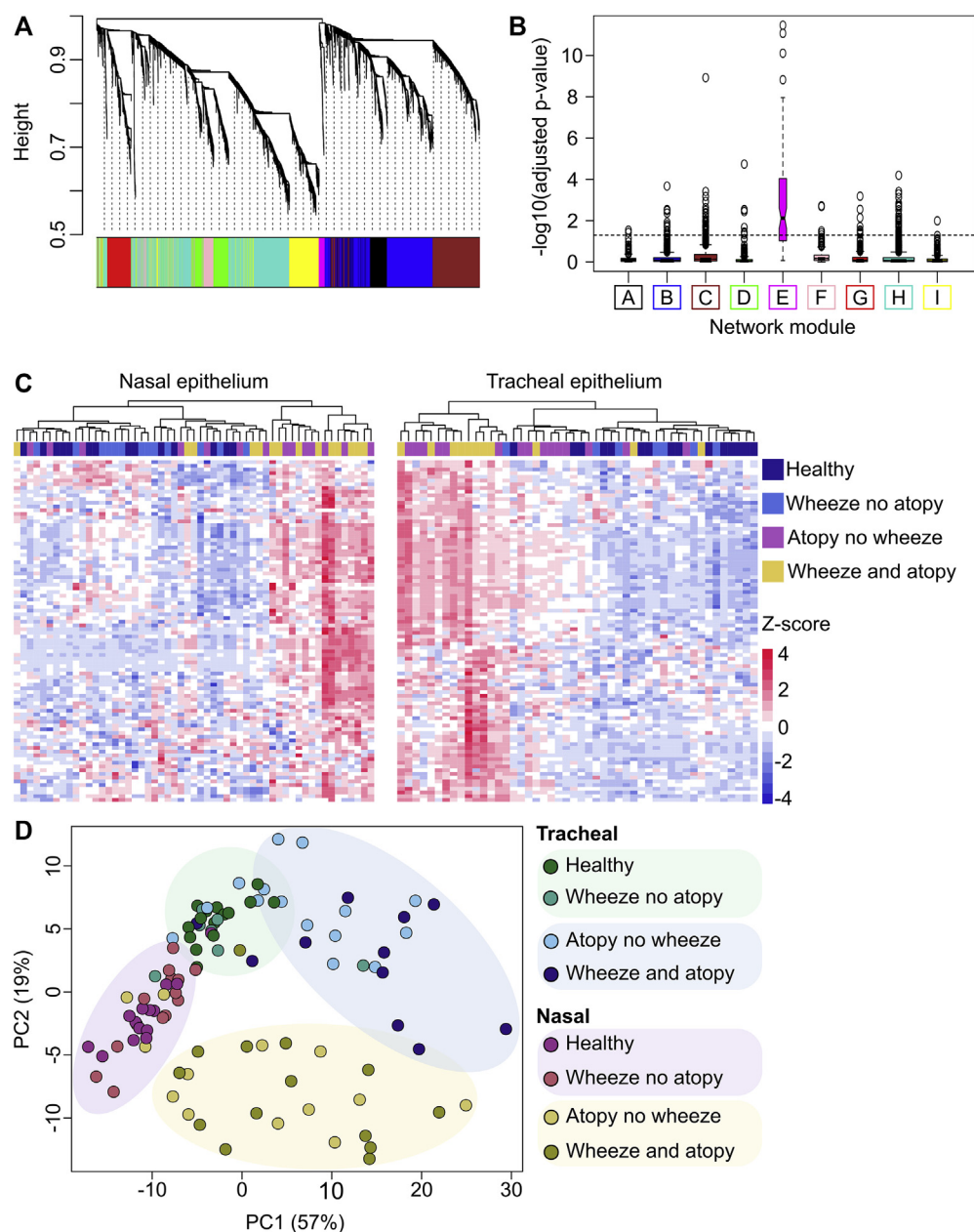


FIG 3. Lower airway disease signature is reflected in the upper airway. **A**, Dendrogram representing the weighted correlation network analysis across tracheal epithelial samples, with each distinct module marked by a unique color. **B**, Each network module was tested for enrichment of differentially expressed genes (between those with atopic asthma and healthy controls within tracheal epithelium), where the horizontal dashed line represents the threshold for significance. Module E was highly enriched for differentially expressed genes. **C**, Heatmaps visualizing expression patterns for the 92 genes within module E across all nasal epithelial samples (left) and tracheal epithelial samples (right), where genes and samples were ranked using unsupervised clustering. **D**, Principal-component analysis across all samples based on the 92 genes within module E.

without wheeze. The results from each of the 4 comparisons across both tracheal and nasal epithelium are summarized in Table IV (see Tables E5-E12 in this article's Online Repository at www.jacionline.org). Comparisons related to the combined effect of atopy and wheeze yielded the largest gene expression differences in both tissues, while overall, disease-associated changes were more prominent in the tracheal epithelium (greater numbers of differentially expressed genes compared with nasal

epithelium). Importantly, we observed a partial overlap between the lists of genes generated from each contrast across tissues, which overall showed strong agreement in terms of fold-change values (see Tables E13-E16 and Fig E5 in this article's Online Repository at www.jacionline.org). The greatest overlap was evident for contrasts between wheeze with atopy and healthy controls; 49 genes were differentially expressed in both the lower and upper airway epithelium, including several well-characterized asthma-

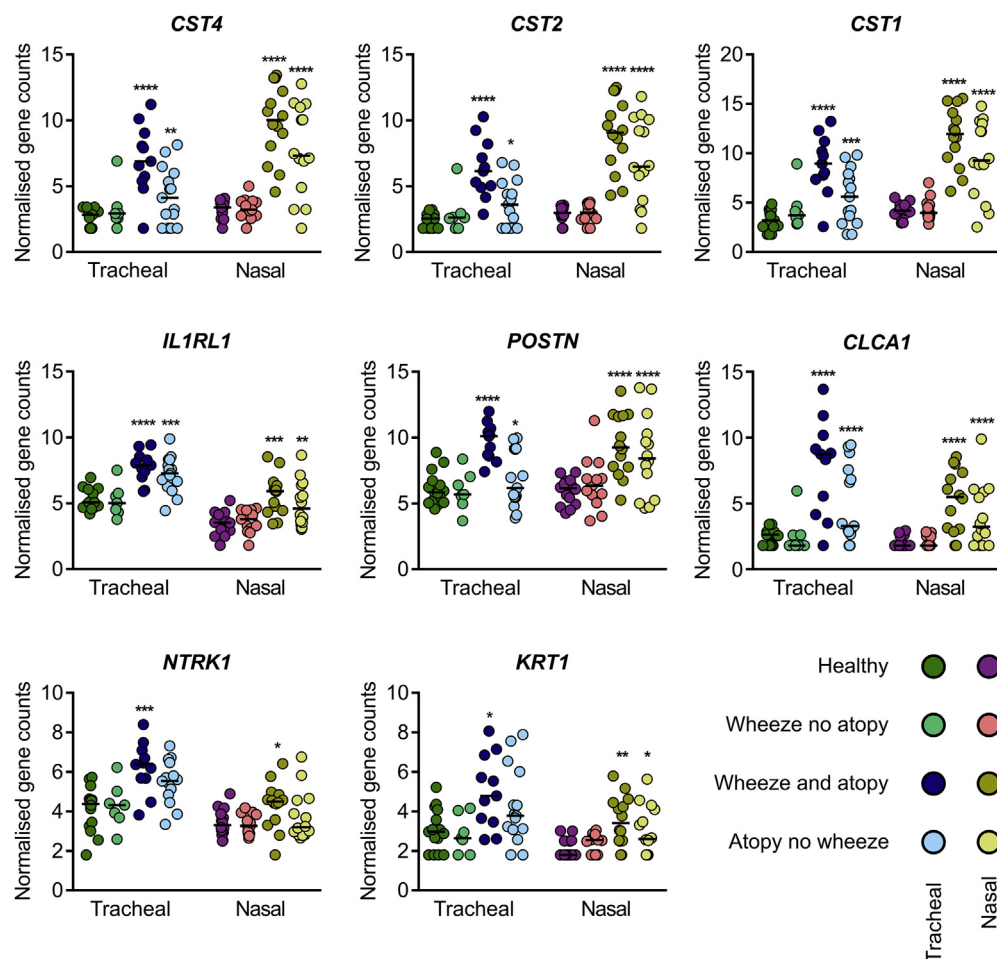


FIG 4. Similar expression of key disease-associated genes within tracheal and nasal epithelium. Normalized gene counts for selected genes representing the most robust gene expression changes across tissues, or hub genes within the protein-protein interaction network for the wheeze with atopy network module. *P* values were derived from DESeq2 analysis for differential gene expression (disease group relative to healthy controls) and adjusted for multiple comparisons using the Benjamini-Hochberg method. Data points represent individual samples, with bars showing median values. **P* ≤ .05, ***P* ≤ .001, ****P* ≤ .0001, *****P* ≤ .00001.

associated genes (IL-1 receptor like 1 [*IL1RL1*], prostaglandin-endoperoxide synthase 1, *CCL26*, and periostin [*POSTN*]).

Lower airway gene signatures in atopic wheezers can distinguish between groups in upper airway samples

We next sought to obtain a more detailed understanding of the molecular mechanisms that characterize the wheeze-associated signatures in the upper and lower airways through weighted gene coexpression network analysis, which provides a holistic view of the functional organization of the gene expression program. Separate networks were constructed for the tracheal and nasal epithelial samples on the basis of genes with significant variation in expression across samples (to capture genes with intrinsic biological variation between phenotypes). The inclusion of a variance-based filter contrasts with the previous network analysis, which was performed on a transcriptome-wide scale. This analysis defined 9 modules (or clusters) of coexpressed genes within the tracheal epithelium (Fig 3, A), and 6 modules within the

nasal epithelium (see Fig E6, A, in this article's Online Repository at www.jacionline.org). To determine which network module(s) was perturbed in relation to wheeze or atopy, each module was checked for enrichment of differentially expressed genes across all 4 contrasts. Module E within the tracheal epithelial network was highly enriched for genes associated with wheeze with atopy, whereas no other network module (in either tissue) was associated with a disease phenotype (Fig 3, B; see Fig E6, B-E). Herein, module E is referred to as the tracheal wheeze with atopy module. To test whether differences in these genes were specific to the lower airway, or were also present in the upper airway, we performed semisupervised clustering analysis using the 92 genes. Heatmap visualization of the 92 genes within this module indicated that overall (1) the genes were more highly expressed in individuals with wheezing with atopy or atopy alone and (2) this expression pattern and sample clustering was consistent within nasal epithelium (Fig 3, C). Importantly, principal-component analysis based on the 92 genes within the tracheal wheeze with atopy module could equally distinguish between groups in nasal epithelium (Fig 3, D). Of note, in both upper and lower airway samples,

subjects with wheeze and atopy or atopy alone tended to cluster together, whereas subjects with wheeze alone clustered more closely with the healthy controls. This is consistent with the fact that none of the genes within the tracheal wheeze with atopy module were significantly differentially expressed in relation to wheeze alone in either tracheal or nasal epithelium (see Table E17 in this article's Online Repository at www.jacionline.org).

Children with atopy exhibit similar gene expression profiles across nasal and tracheal epithelium

To capture the most robust changes associated with wheezers with atopy, the 20 most significantly differentially expressed genes between children with wheeze and atopy and healthy controls, and the 20 genes with the greatest absolute fold-change difference in expression were identified in tracheal and nasal epithelium; that is, genes were ranked once by adjusted *P* value, then again by absolute fold-change for each tissue, resulting in 4 lists. Five genes (cystatin 1 [*CST1*], cystatin 2 [*CST2*], cystatin 4 [*CST4*], chloride channel accessory 1 [*CLCA1*], and *POSTN*) were consistently ranked in the top 20 genes across all 4 lists, all of which were significantly upregulated in children with atopy (Fig 4). In addition, a first-order protein-protein interaction network containing the tracheal wheeze with atopy module genes was created to identify the most highly interconnected genes (hubs; representing key molecules within a network) (see Fig E7 in this article's Online Repository at www.jacionline.org). Again, the atopic groups exhibited increased expression for several hub genes (*IL1R1*, neurotrophic receptor tyrosine kinase 1 [*NTRK1*], and keratin [*KRT1*]) across both nasal and tracheal airway epithelium (Fig 4).

DISCUSSION

The upper and lower respiratory tracts form a continuum allowing the passage of air into and out of the lungs. Despite epidemiological and pathophysiological evidence to suggest a unified airway, there are significant differences between these 2 regions. Although a unified airway has been inferred, there is conjecture on whether this is in fact the case due to a lack of direct evidence. The current study is the largest of its kind conducted to directly test this hypothesis by undertaking a global transcriptomic analysis on freshly isolated nasal and tracheal epithelial cells from children, with and without atopy and/or wheeze. Our observations support the hypothesis of unified airway with 91% homology in the types of genes being expressed in the upper and lower airways. Furthermore, network analysis illustrated conservation of coexpressed (and functionally enriched) gene modules between the upper and lower airways, which were associated with generalized cellular processes including ribosomal function, metabolism, and mitochondrial activity. Furthermore, we identified gene expression changes associated with wheeze and/or atopy (*IL1R1*, prostaglandin-endoperoxide synthase 1, *CCL26*, *POSTN*, *CST1*, *CST2*, *CST4*, *CLCA1*, *NTRK1*) that are inherent to both the upper and lower airway epithelium. Collectively, the findings support the view of a unified airway at the transcriptomic level and provide rationale for future exploration into functional relationships between these 2 regions.

The few studies that have used transcriptomics to compare nasal and tracheal epithelial cells have done so under specific airway disease settings including asthma, cystic fibrosis, and

chronic obstructive pulmonary disease, have used relatively small sample sizes (<15), and compared their data with publicly available transcriptomic data sets.^{33,34} In contrast, our study performed transcriptomic analysis on a pediatric cohort of 63 children with and without wheeze and/or atopy, using predominantly matched nasal and tracheal samples. This study was also designed and conducted with high stringency with only those RNA samples meeting the set quality (ratio, >1.80), integrity (RIN, >7.8), and quantity (>800 ng) standards subsequently sequenced. Procedures were also harmonized for nasal and airway sampling as well as the processing and extensive training at the initial phase of the study ensured that the method and location of sampling as well as the subsequent processing was standardized. We have also minimized sampling and technique confounders to the study by using only 1 physician to obtain all samples using the 1 nylon cytology brush. As a result, we feel that this study has a unique robustness that has enabled detailed and meaningful analysis of its findings.

Not surprisingly, we identified a family of keratin genes (*KRT23*, *KRT33A*, *KRT34*, *KRT36*, *KRT37*, *KRT71*) that were not differentially expressed between sites. Being archetypal lineage markers, broad commonality between the nasal and tracheal epithelium of these genes supports the concept of a common cell lineage and unified airway. Furthermore, observed similarities between the nasal and tracheal epithelium in terms of modules/clusters of coexpressed genes highlights the globally conserved biological functions across both types of epithelium irrespective of disease phenotype, although the higher number of modules initially defined in tracheal epithelium may reflect a higher level of functional complexity in the lower airway. For example, several tight junction gene expression levels (cadherin [*CDH10*], *CDH15*, *CDH19*, *CDH9*, protocadherin 11 X-linked, protocadherin 8) were similarly expressed in both the upper and lower airways and suggest a commonality in barrier function maintenance.

Further exploration of the genes more highly expressed in the upper airway found that in addition to olfactory genes (*ORFE129P*, *ORF7E158P*, *ORF7E91P*, *ORF7E99P*) and genes related to epithelial lineage (*KRT16*, *KRT16P3*, *KRT18P32*, *KRT8P5*, *KRT2*, *KRT24*, *KRT8P1*, *KRT8P38*, *KRTP5-9*) were genes from the uridine 5'-diphospho-glucuronosyltransferase family (*UGT1A10*, *UGT1A7*, *UGT1A8*, *UGT1A2*, *UGT1A3*, *B3GALT1*, *B3GNT6*, *GALNT13*, *GALNT16*, *GALNT4*). These genes have been found to be expressed in the lung epithelium^{35,36} in addition to hepatic tissue and collectively act to remove toxins, endogenous substances, and foreign chemicals, which align to the primary function of the upper airway.^{37,38} Tracheal epithelial cells on the other hand were found to more highly express tubulin genes (*TUBAL3*, *TUBB8P7*) whose purpose is to direct airway ciliary function.^{39,40} This is corroborated further by the observation that network modules related to cilium assembly/activity were more highly expressed in the lower airway. Also prominent in the lower airway were genes related to calcium-activated chloride channels (*ANO3*, *ANO4*, *ANO5*, *BEST3*, *CADPS*, *CACNG4*, *CACNA1B*, *CACNA1E*, *CACNA1H*), which regulate and maintain the air surface liquid layer at the luminal surface of epithelia, which is critical for effective mucociliary clearance.^{41,42} We then broadened our comparisons to include the skin to determine whether the predominantly unified signature extended beyond the airway into other epithelial tissues. We identified a lower level of similarity

(75%) in the genes expressed in the skin and the nasal/tracheal epithelium compared with the similarity observed between nasal and tracheal epithelium (95%), which was corroborated by weaker correlations observed in the gene expression profiles between the 2 sites. Collectively, these results suggest that although the upper and lower airways exhibit conservation of gene expression for most genes, differential regulation of gene expression affecting a small subset of genes is evident between the 2 sites, reflecting site-specific functions. Furthermore, and probably not surprisingly, our data show that even though there is overlap in the gene signature patterns between respiratory and nonrespiratory epithelium, there are unique features in the airway that do not extend to other types of epithelium.

We believe this is the largest study of its kind to compare transcriptional profiles of airway epithelium between children who exhibit wheeze and atopy using paired nasal and tracheal primary airway epithelial samples. Our finding of a conserved signature across both epithelial tissues in children with wheeze and atopy supports other investigations observing similar transcriptional profiles between nasal and tracheal tissues in the context of lower airway pathologies including childhood asthma,^{43,44} chronic obstructive pulmonary disease,⁴⁵ and those induced through cigarette smoke.^{46,47}

Interestingly, several identified genes in our wheeze with atopy group have been also associated with lower airway pathology, namely, asthma. Considering that the processes that lead to asthma development start very early in life and persistent wheezing combined with allergies is a strong risk factor for its development, overlaps in the expression of particular genes may be expected. Of relevance to this study are the 5 genes representing the most robust changes in expression associated with wheeze and atopy across both nasal and tracheal epithelium (*CST1*, *CST2*, *CST4*, *CLCA1*, and *POSTN*). *CST1*, *CST2*, and *CST4* encode for the closely related cystatin-S proteins⁴⁸ and have previously been reported as significantly upregulated in bronchial epithelium of patients with mild to moderate asthma,⁴⁹ and in nasal epithelium from individuals with allergic rhinitis (*CST1* only⁵⁰). Cystatins are a large family of peptides that are potent inhibitors of cysteine proteinases and are often referred to as “salivary cystatins” due to their recognized role as defense proteins within the saliva,⁵¹ even though cystatin-S proteins are also expressed in both bronchial and nasal lavage fluids.⁵² Another identified gene, *CLCA1* (chloride channel accessory 1), belongs to a family of calcium-sensitive chloride conductance proteins and as such is a critical physiological regulator of chloride transport and mucin expression; *CLCA1* is highly upregulated in response to IL-13 and can drive mucus production in human AECs.⁵³ The role of *CLCA1* in hypersecretory respiratory diseases including asthma, chronic obstructive pulmonary disease, and cystic fibrosis is well documented.^{54,55} Finally, *POSTN* encodes for periostin, a secreted extracellular matrix protein that is a well-investigated marker of airway remodeling.⁵⁶ *POSTN* expression is induced downstream of IL-4/IL-13 signaling in human AECs⁵⁷ and plays a role in wound healing and profibrogenic processes.⁵⁸ Importantly, overexpression of *POSTN* delays wound healing in mice⁵⁹ and promotes airway hyperresponsiveness and inflammation.⁶⁰ Collectively, the fact that the conserved epithelial signature associated with wheeze and atopy identified in this study contains many genes previously identified as relevant to asthma pathogenesis highlights the clinical relevance of our findings.

Several other genes within our conserved epithelial signature are hallmarks of T_H2-driven inflammation often observed in asthma or are genes in which genetic variation is significantly associated with asthma risk (*CCL26*, *IL1R1*, and *NTRK1*).^{56,61-63} Together, these results suggest that there is a common mechanism underlying the intrinsic changes that extend between the upper and lower airway epithelium in children with atopy and asthma, and although the differences may affect only a subset of genes (49 in total), they represent functionally important changes that are directly relevant to lower airway pathology.

Our study is not without limitations. First, our study samples were collected from a community cohort, and although subgroups could be identified, better definition of these using strong accompanying criterion would strengthen the ability to compare results between these groups. Second, our sampling methodology obtained epithelial cells from the central airway, namely, just above the carina. We were unable to sample deeper in the lung and thus our interpretations of a “unified airway” are supportive of but limited to the central airway. Future studies may explore testing the hypothesis and comparing nasal and peripheral bronchiolar epithelium. Third, although our sampling methodology yields a more than 95% pure epithelial cell population, we acknowledge that our samples may not be entirely pure. Assessments of collected samples have identified a small population of macrophages (1%-5% of sample) as well as a heterogeneous epithelial cell population consisting of both terminally differentiated epithelial cells as indicated by the presence of cilia and non-ciliated basal epithelial cells.¹⁹ Our routine sample processing methodology positively selects out the macrophages,^{18,19} and interrogation of resulting cell populations has confirmed that samples are not contaminated by other cell types, including mesenchymal cells, macrophages, dendritic cells, and endothelial cells.¹⁸ Although these have been excluded, we recognize that we may potentially have other cell types that would still be detected by RNA sequencing. This is particularly so for immune cells including lymphocytes and monocytes, which are known to have well-characterized pathways/gene ontologies.^{64,65} In fact, modules identified as part of this study included pathways involved in T-cell signaling and B-cell signaling. Although beyond the scope of the current study, future experiments could use single-cell sequencing to delineate the exact transcriptomic signature for each cell type in the airway epithelium.^{66,67} Finally, atopy was determined in this study using a radioallergosorbent test. This was justified because it was directly measured at time of recruitment and additional information such as eczema and hay fever was only parent reported. Future studies would be strengthened if this information was confirmed as current manifestations and combined with radioallergosorbent test results to further define the atopy group.

Although data generated from this study indicate primary homology between the upper and lower airways, the importance of the unified airway hypothesis lies in its approach to therapy. The hypothesis assumes that commonalities between the nasal and tracheal mucosa are functionally complementary and thus can be treated in a unified fashion. Our observations suggest that this is the case, with biological conservation evident. Considering, because clinical evidence suggests that treating upper airway diseases such as rhinitis benefits the lower airway,⁶⁸⁻⁷⁰ there is inference that certain functional complementarity between the nose and the lower airway may facilitate a unified approach to treatment.

Conclusions and future directions

Collectively, this study, the largest of its kind, has used transcriptomics to comprehensively and systematically compare upper and lower AECs. Data generated identified 91% homology, with the remainder being uniquely expressed to each region and associated with site-specific roles. There exists enormous interest as to whether nasal cells can act as an informative surrogate for the lower airway. Our findings suggest that this may be the case but dependent on whether both sites are affected to a similar extent and whether both translate to similar functional outcome. With significant clinical interest also in biomarker discovery, and the use of nasal cells as an easily accessible surrogate for lower airway tissues, the conserved epithelial signature identified in this study holds promise for development into a screening tool. Although further work is required to determine the predictive value of these genes as biomarkers for chronic airway diseases, this unique data set provides a valuable resource for future meta-analyses or integrated -omic studies.

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Key messages:

- Nasal and tracheal epithelium exhibit similar transcriptional profiles, with some minor differences reflective of site-specific function.
- Gene expression changes associated with wheeze and atopy are reflected in both tracheal and nasal epithelium.
- These changes include some genes that are well characterized in the context of asthma (*CLCA1*, *IL1RL1*, *CCL26*, and *POSTN*).

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