

Identification of airway mucosal type 2 inflammation by using clinical biomarkers in asthmatic patients

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Background: The Airways Disease Endotyping for Personalized Therapeutics (ADEPT) study profiled patients with mild, moderate, and severe asthma and nonatopic healthy control subjects.

Objective: We explored this data set to define type 2 inflammation based on airway mucosal IL-13–driven gene expression and how this related to clinically accessible biomarkers.

Methods: IL-13–driven gene expression was evaluated in several human cell lines. We then defined type 2 status in 25 healthy subjects, 28 patients with mild asthma, 29 patients with moderate asthma, and 26 patients with severe asthma based on airway mucosal expression of (1) CCL26 (the most differentially expressed gene), (2) periostin, or (3) a multigene IL-13 *in vitro*

signature (IVS). Clinically accessible biomarkers included fraction of exhaled nitric oxide (FENO) values, blood eosinophil (bEOS) counts, serum CCL26 expression, and serum CCL17 expression.

Results: Expression of airway mucosal CCL26, periostin, and IL-13–IVS all facilitated segregation of subjects into type 2–high and type 2–low asthmatic groups, but in the ADEPT study population CCL26 expression was optimal. All subjects with high airway mucosal CCL26 expression and moderate-to-severe asthma had FENO values (≥ 35 ppb) and/or high bEOS counts (≥ 300 cells/mm³) compared with a minority (36%) of subjects with low airway mucosal CCL26 expression. A combination of FENO values, bEOS counts, and serum CCL17 and CCL26 expression had 100% positive predictive value and 87% negative predictive value for airway mucosal CCL26–high status. Clinical variables did not differ between subjects with type 2–high and type 2–low status. Eosinophilic inflammation was associated with but not limited to airway mucosal type 2 gene expression.

Conclusion: A panel of clinical biomarkers accurately classified type 2 status based on airway mucosal CCL26, periostin, or IL-13–IVS gene expression. Use of FENO values, bEOS counts, and serum marker levels (eg, CCL26 and CCL17) in combination might allow patient selection for novel type 2 therapeutics. (*J Allergy Clin Immunol* 2017;■■■:■■■–■■■.)

Key words: Asthma, type 2 inflammation, phenotypes, airway mucosal gene expression, biomarkers

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Asthma is a heterogeneous disease characterized by chronic airway inflammation.¹ Although there are many proposed asthma phenotypes, the underlying phenotypes are poorly understood and hard to identify, making tailored asthma treatment an even greater challenge.

Type 2 inflammation in asthmatic patients (also termed T_H2 inflammation) refers to inflammation driven by type 2 cytokines, such as IL-4, IL-5, and IL-13, secreted by CD4⁺ T_H2 cells, as recently reviewed.² Type 2 innate lymphoid cells have also been proposed to secrete the same cytokines.³ Single and multiple gene signatures in airway brushings and sputum inflammatory cells have recently been used to define type 2–high or type 2–low status.^{4–6}

The most effective therapies for asthma are anti-inflammatory, including inhaled corticosteroids (ICSs) and mAbs, but these do not work in all patients. For example, the response to ICSs is

Abbreviations used

ADEPT:	Airways Disease Endotyping for Personalized Therapeutics
bEOS:	Blood eosinophil
CLCA1:	Calcium-activated chloride channel regulator 1
FDR:	False discovery rate
FENO:	Fraction of exhaled nitric oxide
ICS:	Inhaled corticosteroid
IVS:	<i>In vitro</i> signature
NPV:	Negative predictive values
PPV:	Positive predictive value
sCCL17:	Serum CCL17
sCCL26:	Serum CCL26
SERPINB2:	Serpin peptidase inhibitor, clade B, member 2 (also known as plasminogen activator inhibitor 2)
sIgE:	Serum IgE
spEOS:	Sputum eosinophil

associated with a high fraction of exhaled nitric oxide (FENO) value,⁷ high sputum eosinophil (spEOS) count,⁸ or 3-gene type 2–high signature in epithelial brushings.⁵ Similarly, for the mAbs omalizumab (anti-IgE),⁹ dupilumab (anti-IL-4 receptor),¹⁰ mepolizumab and reslizumab (anti-IL-5),^{11,12} or lebrikizumab (anti-IL-13),¹³ optimal efficacy is seen in patients with severe asthma with type 2 inflammation identified based on biomarkers, including blood eosinophil (bEOS) counts, spEOS counts, FENO values, or serum periostin expression.^{9–13}

Notably, despite these patient selection strategies, severe asthma is often incompletely responsive to these and other currently available therapies.^{14,15} This might be due to difficulty in defining phenotypes, including the type 2 phenotype, leading to selection of inappropriate therapy or additional mechanisms alone or in addition to type 2 inflammation, which are driving pathology. For example, in a recent report for lebrikizumab, even subjects with high periostin levels had a 95% confidence limit range for improvement in FEV₁ of between 1.0% and 15.4%,¹³ illustrating that single biomarkers cannot capture complex biological networks.

The previously reported Airways Disease Endotyping for Personalized Therapeutics (ADEPT) study¹⁶ allowed us to evaluate airway mucosal gene expression associated with type 2 inflammation, including periostin expression, which has been associated with enhanced responses to the anti-IL-13 mAb lebrikizumab¹³; CCL26, which in our hands was the most highly expressed gene in IL-13–treated human cells lines; and a multigene *in vitro* signature (IVS) from the same cell lines (IL-13–IVS). We also ascertained whether type 2 gene expression profiles for CCL26, IL-13–IVS, and periostin could be classified by using clinically accessible biomarkers, namely FENO values, bEOS counts, and less commonly used serum markers, such as CCL17/TARC (thymus and activation-regulated chemokine) and activation-regulated chemokine and CCL26/eotaxin-3, which are type 2 chemokines reliably measured in serum.

METHODS**Study design and population**

The ADEPT study design and population (clinicaltrials.gov registration no. NCT01274507) have been described in detail.¹⁶ In brief, nonatopic healthy subjects, patients with mild asthma (no controller medications and

prebronchodilator FEV₁ ≥80% of predicted value), patients with moderate asthma (low-to-moderate ICS dose and prebronchodilator FEV₁ 60% to <80% of predicted value), and patients with severe asthma (high-dose ICS and prebronchodilator FEV₁ 50% to less than 80% of predicted value) were enrolled. Healthy subjects and a subset of asthmatic patients underwent bronchoscopy. All ADEPT study subjects with good-quality airway mucosal tissue from biopsy specimens were included in the biopsy assessment detailed below. Clinical assessments included spirometry, bronchodilator reversibility, methacholine airway hyperresponsiveness, the Asthma Control Questionnaire,¹⁷ and the Asthma Quality of Life Questionnaire.¹⁸ The study was approved by site-specific institutional review boards, and all subjects signed an informed consent form. Healthy and asthmatic subjects attended screening, baseline, and bronchoscopy visits, and asthmatic patients also attended biomarker visits at 3, 6, and 12 months.

Biomarker assessments

Bioanalysis methods are detailed in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Briefly, gene expression in RNAlater-preserved biopsy samples was analyzed by means of microarray (HG-U133+PM platform; Affymetrix, Santa Clara, Calif), and histology of biopsy specimens was performed by Pantomics (Richmond, Calif). Induced sputum was collected, processed by using the plug selection method,¹⁹ and analyzed for differential cell counts. Biomarker analyses focused on molecular type 2 activity status in biopsy specimens as they relate to asthma characteristics and clinical biomarkers.

Definition of type 2 status in biopsy specimens

Type 2–high versus type 2–low phenotypes were defined *a priori* based on inferred IL-13 activity in airway mucosal biopsy specimens from asthmatic patients compared with those from healthy control subjects by using gene expression of CCL26, periostin, or a multigene in-house IL-13–IVS (see the [Methods](#) section and [Table E1](#) in this article's Online Repository at www.jacionline.org). IL-13 activity was selected because it is the most broadly expressed type 2 cytokine produced by T_H2 cells²⁰ and also type 2 innate lymphoid cells.²¹ CCL26 mediates eosinophil infiltration into the airway and is the most highly expressed selective IL-13–driven gene.^{22–24} The chemokine CCL17 is induced by IL-13^{24,25} and decreased by anti-IL-13 and anti-IL-4 receptor mAb therapeutics.^{10,13,26} For CCL26, the highest signals in the healthy control subjects were just below the limit of reliable quantification for the microarray, and therefore this limit of quantification (log₂ intensity of 5.0) was set as the threshold for airway CCL26–high versus airway CCL26–low status. For periostin and IL-13–IVS, type 2–high status was defined as gene expression (or enrichment) beyond the 95th percentile of the healthy control subjects.

Clinical biomarkers evaluated for their association with type 2–high or type 2–low status by airway IL-13–driven gene expression included FENO values, bEOS counts, spEOS counts, serum IgE levels, and expression of 2 serum type 2–associated biomarkers, serum CCL17 (sCCL17) and serum CCL26 (sCCL26). Details of the assays can be found in the [Methods](#) section in this article's Online Repository. Expression of sCCL17 and sCCL26 was measured repeatedly over 12 months to assess stability.

Statistical analysis

Healthy subjects (n = 25) and non-ICS-treated patients with mild asthma (n = 28) were analyzed separately, whereas the ICS-treated moderate (n = 29) and severe (n = 26) asthma cohorts were pooled. We focused analyses on the patients with moderate-to-severe asthma because they have unmet clinical need despite ICS treatment. Separate analyses on the moderate and severe asthma cohorts were not undertaken because of limited statistical power.

Statistical analyses used Array Studio (version 7; OmicSoft, Cary, NC; www.omicsoft.com). For data with log-normal distributions, logarithmic transformations were performed. Group comparisons of gene and protein expression levels (log₂-transformed) were performed by using general linear

models. For variables with log-normal distributions, geometric means and asymmetric SDs were estimated from the log₂-scaled mean and SD as follows:

$$\text{Geometric mean} = 2^{\wedge}(\text{mean});$$

$$\text{Asymmetric upper SD} = 2^{\wedge}(\text{mean} + \text{SD}) - 2^{\wedge}(\text{mean});$$

$$\text{Asymmetric lower SD} = 2^{\wedge}(\text{mean} - \text{SD}) - 2^{\wedge}(\text{mean}).$$

Significance of differences in proportions between 2 categorical variables was tested by using the Fisher exact test. Nominal *P* values are reported, with multiple testing-adjusted false discovery rate (FDR) values of less than 0.05 indicated. Classification model statistics were obtained from logistic regression and receiver operating characteristic analyses (NCSS, version 8; www.ncss.com).

Box-and-whisker plot representations of distributions in figures show the median and interquartile range (box), minimum and maximum range (whiskers), and mean (+ symbol), and diamond symbols show the values for each subject/sample.

RESULTS

Airway mucosal CCL26, periostin, and IL-13-IVS gene expression compared for classification of type 2 inflammation

Fig 1 displays segregation into high and low type 2 status of asthmatic patients from healthy subjects based on airway mucosal gene expression of CCL26 (Fig 1, A), periostin (Fig 1, B), or IL-13-IVS enrichment (Fig 1, C). Asthmatic patients with high airway mucosal CCL26 expression had mostly high airway periostin (90%) and airway IL-13-IVS (83%) expression (all asthmatic patients combined), a finding unaffected by asthma severity (Table I and see Fig E1 in this article's Online Repository at www.jacionline.org). Lower proportions of asthmatic patients with high airway periostin expression had high airway CCL26 (62%) or airway IL-13-IVS (67%) expression for all asthmatic patients combined, but the concurrence was higher in those with mild compared with moderate-to-severe asthma (Table I and see Fig E1). Similarly, lower proportions of asthmatic patients with high airway IL-13-IVS expression had high airway CCL26 (62%) or airway periostin (67%) expression for all asthmatic patients, with higher concurrence with mild compared with moderate-to-severe asthma (Table I and see Fig E1).

These observations drove selection of airway mucosal CCL26 expression as the primary anchor for type 2 status, although periostin and IL-13-IVS expression also demonstrated utility for segregation of asthmatic patients from healthy control subjects, as discussed in the Results section (also see Fig E1) in this article's Online Repository at www.jacionline.org.

Demographics by airway mucosal CCL26 status

Table II presents demographic characteristics for patients with mild and moderate-to-severe asthma by high and low airway mucosal CCL26 status. There were no significant differences by CCL26 status. Similar proportions of CCL26-high (3/13 [23%]) and CCL26-low (7/42 [17%]) status subjects used leukotriene inhibitors (data now shown).

Asthma disease characteristics and clinical biomarkers by airway CCL26 status

There were decreasing proportions of subjects with high airway CCL26 expression in the mild (*n* = 16/28 [57%]), moderate (*n* = 8/29 [28%]), and severe (*n* = 5/26 [19%]) asthma cohorts.

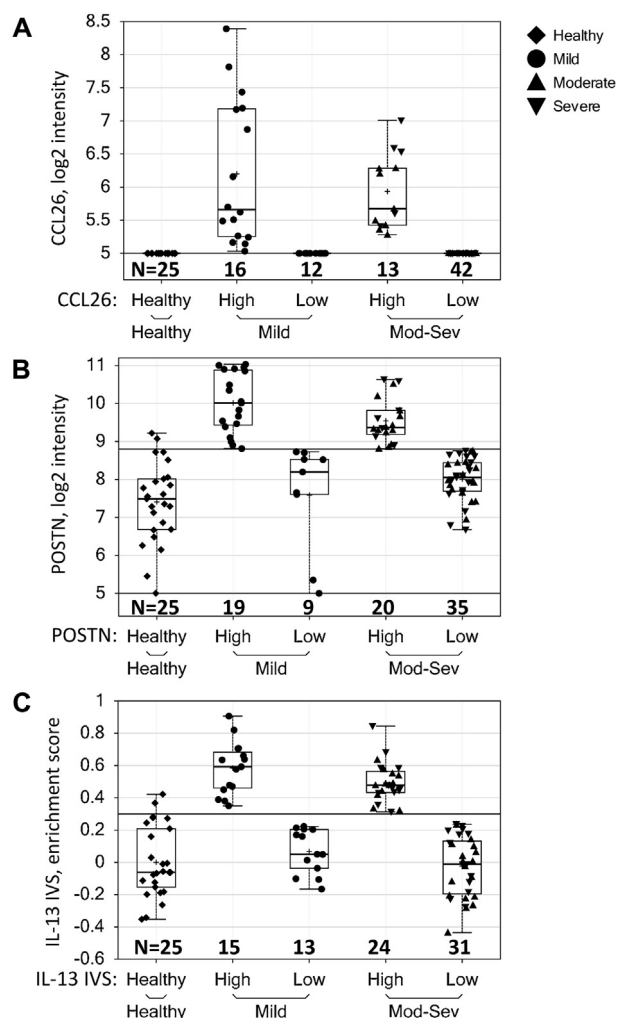


FIG 1. Airway mucosal expression-based categorization of the type 2 phenotype. Airway mucosal CCL26 (A) and periostin (POSTN; B) gene expression (log₂ intensity) and IL-13-IVS enrichment (gene set variation analysis scores; C) are presented in the healthy or mild and moderate-to-severe asthma cohorts stratified by airway mucosal type 2-high versus type 2-low status (x-axis, top label), as defined by CCL26 (Fig 1, A), periostin (Fig 1, B), or IL-13-IVS (Fig 1, C) expression. The horizontal solid line indicates the threshold for the high phenotype.

Disease characteristics, including airflow obstruction, were not associated with airway CCL26 status (Table III), except that CCL26-high status was associated with nominally significantly increased airway hyperresponsiveness in patients with mild asthma (*P* = .045). In contrast, airway mucosal CCL26 status was strongly associated with multiple type 2 clinical biomarkers. Thus FENO values (all asthma severities) and bEOS counts (moderate-to-severe asthma) were significantly increased (*P* < .05) in the airway mucosal CCL26-high group compared with the airway mucosal CCL26-low group (Fig 2, A and B, and Table III). Serum IgE levels were increased greatly in the airway mucosal CCL26-high group with moderate-to-severe asthma (*n* = 13) compared with the healthy control group (FDR < 0.0001, *P* < .0001, 24-fold) and the airway mucosal CCL26-low group (*n* = 41, FDR = 0.091, *P* = .018, 2.8-fold), although sIgE levels were also increased in the airway mucosal CCL26-low group compared with the healthy control group

TABLE I. Segregation of airway mucosal type 2–high versus airway mucosal type 2–low status by airway mucosal CCL26, periostin, and IL-13–IVS gene expression

	Mild asthma			Moderate-to-severe asthma		
	Airway mucosal CCL26 high	Airway mucosal periostin high	Airway mucosal IL-13–IVS high	Airway mucosal CCL26 high	Airway mucosal periostin high	Airway mucosal IL-13–IVS high
Airway mucosal CCL26 high	100%*	94%	81%	100%	85%	85%
Airway mucosal CCL26 low	0%	33%	17%	0%	21%	31%
Airway mucosal periostin high	79%	100%	68%	55%	100%	65%
Airway mucosal periostin low	11%	0%	22%	6%	0%	31%
Airway mucosal IL-13–IVS high	87%	87%	100%	46%	54%	100%
Airway mucosal IL-13–IVS low	23%	46%	0%	6%	23%	0%

*Percentage of patients with mild or moderate-to-severe asthma within the airway group indicated in rows with airway mucosal–high status based on biomarkers indicated in columns.

TABLE II. Demographics by airway mucosal CCL26 expression status for the mild and moderate-to-severe asthma groups

	Mild asthma			Moderate-to-severe asthma		
	Airway mucosal CCL26 high (n = 16)	Airway mucosal CCL26 low (n = 12)	P value*	Airway mucosal CCL26 high (n = 13)	Airway mucosal CCL26 low (n = 42)	P value*
Age (y), mean ± SD (range)	27.0 ± 7.6 (21.0–49.0)	30.4 ± 12.2 (18.0–54.0)	.37	42.8 ± 9.4 (26.0–54.0)	41.2 ± 11.3 (18.0–55.0)	.66
BMI (kg/m ²), mean ± SD (range)	24.6 ± 3.0 (19.6–29.6)	24.1 ± 3.4 (20.2–30.1)	.69	26.9 ± 2.9 (21.4–31.2)	26.5 ± 3.8 (19.1–32.1)	.71
Duration of asthma (y), mean ± SD (range)	16.6 ± 6.9 (7.1–31.6)	15.7 ± 8.7 (0.7–32.9)	.76	21.6 ± 10.7 (3.6–39.5)	19.2 ± 12.8 (0.3–47.5)	.55
Male sex, no./total (% of total)	8/16 (50%)	3/12 (25%)	.25	8/13 (62%)	16/42 (38%)	.20
Race, no./total (% of total)			.50			1.00
White	14/16 (88%)	12/12 (100%)		10/13 (77%)	31/42 (74%)	
Black	0/16 (0%)	0/12 (0%)		2/13 (15%)	8/42 (19%)	
Other	2/16 (13%)	0/12 (0%)		1/13 (8%)	3/42 (7%)	

BMI, Body mass index.

*P values for comparison between airway mucosal CCL26–high and airway mucosal CCL26–low groups within the mild or moderate-to-severe asthma populations.

(FDR < 0.0001, $P < .0001$, fold = 8.6; Fig 3, A). sCCL17 expression was significantly increased in the airway mucosal CCL26–high group compared with the airway mucosal CCL26–low group ($P = .0039$, 2.17-fold/type 2 low) and the healthy control group ($P = .018$, 2.02-fold/healthy control subjects) for patients with moderate-to-severe but not mild asthma ($P = .29$ and $.90$, respectively; Fig 3, B). Similarly, sCCL26 expression was significantly increased in the airway mucosal CCL26–high group compared with the airway mucosal CCL26–low group ($P = .0027$, 7.35-fold/type 2 low) and compared with healthy control subjects ($P = .029$, 4.67-fold/healthy control subjects) for patients with moderate-to-severe but not mild asthma ($P = .21$ and $.67$, respectively; Fig 3, C).

Categorical analysis of clinical biomarkers by airway mucosal CCL26 status

Airway mucosal CCL26 status was compared based on clinical biomarkers categorized by using the following cutoffs: 35 ppb or greater for FENO values, a previously described cutoff^{27–30}; 300 cells/mm³ or greater for bEOS counts, a cutoff used for novel “type 2/eosinophilic” therapeutics³¹; and 95th percentile of the healthy control cohort for sIgE levels (Table IV). sCCL17-high and sCCL26-high status were based on the 75th percentile of the healthy control subjects rather than the 95th percentile because of the wide distributions in healthy control subjects.

A majority of patients with moderate-to-severe asthma with high airway CCL26 expression had high FENO values (69%) compared with 24% of subjects with low airway CCL26

expression ($P = .0063$). Similarly, 77% of subjects with high airway CCL26 expression had high bEOS counts compared with 24% of subjects with low airway CCL26 expression ($P = .0009$). All subjects with high airway CCL26 expression had high FENO values or bEOS counts, but only 55% had high FENO values and high bEOS counts. In contrast, for subjects with low airway CCL26 expression, 36% had high FENO values or high bEOS counts, but few (12%) had high FENO values and high bEOS counts ($P < .0001$ and $.014$, respectively, for high versus low airway CCL26 expression). Of note, 92% of subjects with high airway CCL26 expression had high sIgE levels, but 71% of subjects with low airway CCL26 expression also had high sIgE levels ($P = .15$). For patients with moderate-to-severe asthma, 11 (52%) of 21 had high sCCL17 expression, but only 2 (6%) of 31 asthmatic patients with low CCL17 expression had high airway CCL26 expression. Additionally, 7 (64%) of 11 had high sCCL26 expression, but only 5 (12%) of 42 asthmatic patients with low sCCL26 expression had high airway CCL26 expression.

Similar associations for mild asthma (Table III and see the Results section in this article’s Online Repository) were observed as for moderate-to-severe asthma, except that both the group with high airway CCL26 expression and the group with low expression had mostly low bEOS counts among those with mild asthma (75% and 92% of the respective groups).

In general, the patterns of categorical clinical biomarkers reported for airway mucosal CCL26 expression were similar for periostin (see Table E2) and IL-13–IVS expression (see Table E3 in this article’s Online Repository at www.jacionline.org), although the

TABLE III. Asthma disease characteristics and clinical biomarkers by airway mucosal CCL26 expression status

	Mild asthma			Moderate-to-severe asthma		
	Airway mucosal CCL26 high	Airway mucosal CCL26 low	<i>P</i> value†	Airway mucosal CCL26 high	Airway mucosal CCL26 low	<i>P</i> value†
FEV ₁ , prebronchodilator (PN)	93.6 ± 11.7 [15]	90.8 ± 14.3 [12]	.577	70.2 ± 10.3 [13]	71.2 ± 11.6 [41]	.785
FEV ₁ , postbronchodilator (PN)	102.1 ± 9.1 [15]	97.9 ± 11.2 [12]	.293	83.0 ± 8.9 [13]	80.8 ± 13.8 [41]	.598
BDR, % change in FEV ₁	6.25 ± 8.54 [15]	6.33 ± 3.25 [12]	.978	19.79 ± 15.86 [13]	14.63 ± 10.96 [40]	.195
ACQ score	0.6 ± 0.5 [16]	0.9 ± 0.6 [12]	.176	1.7 ± 0.8 [13]	1.5 ± 0.9 [42]	.549
AQLQ score	6.2 ± 0.7 [16]	5.9 ± 0.9 [12]	.245	5.6 ± 0.8 [13]	5.5 ± 1.3 [42]	.637
PC ₂₀ (mg/mL)*	0.84 +5.33/−0.73 [16]	3.33 +8.18/−2.37 [12]	.045	0.32 +1.77/−0.27 [10]	0.95 +4.76/−0.79 [35]	.102
FENO (ppb)*	52.8 +44.0/−24.0 [16]	27.4 +17.7/−10.8 [12]	.0051	56.8 +56.7/−28.4 [13]	24.9 +23.7/−12.1 [41]	.0003
bEOS (1000/mm ³)*	0.21 +0.19/−0.10 [16]	0.12 +0.13/−0.06 [12]	.051	0.36 +0.22/−0.14 [13]	0.19 +0.20/−0.10 [42]	.0032
sIgE (RFU)*	17.1 +33.2/−11.3 [16]	5.4 +21.8/−4.3 [12]	.032	20.0 +27.4/−11.6 [13]	7.8 +25.9/−6.0 [41]	.032
sCCL17 (pg/mL)*	169 +226/−97 [16]	117 +103/−55 [12]	.14	398 +299/−171 [13]	169 +236/−98 [42]	.0006
sCCL26 (pg/mL)*	117 +732/−101 [16]	42 +127/−32 [9]	.185	416 +3301/−369 [12]	57 +290/−48 [41]	.0023
spEOS (% WBC)*	1.2 +8.4/−1.1 [12]	0.5 +1.1/−0.3 [7]	.291	6.8 +12.9/−4.5 [8]	2.2 +12.4/−1.9 [31]	.117
spNEU (% WBC)	45.6 ± 35.6 [12]	52.1 ± 41.1 [7]	.561	50.8 ± 35.1 [8]	46.9 ± 25.4 [31]	.893

Values are presented as means ± SDs [no.].

AQLQ, Asthma Quality of Life Questionnaire; BDR, Bronchodilator reversibility; PN, predicted normal; RFU, relative fluorescence units.

*Geometric mean ± asymmetric SD [no.]. *P* values were calculated from log-transformed data.

†*P* values for comparison between the airway mucosal CCL26-high and airway mucosal CCL26-low groups within the mild or moderate-to-severe asthma populations. Values are shown in boldface at an FDR of less than 0.05.

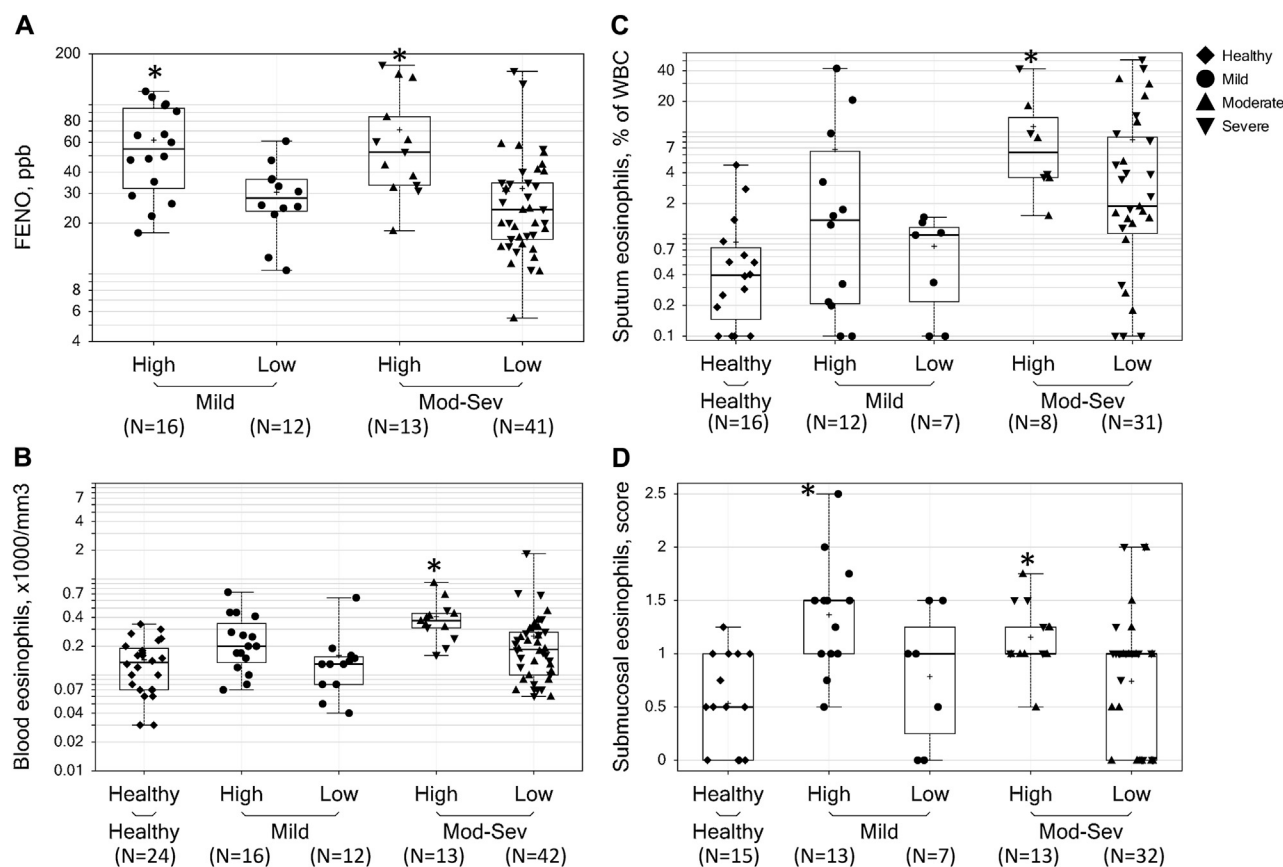


FIG 2. Top associated clinical and biomarker variables to airway mucosal CCL26 expression status. Screening/baseline values for FENO (**A**), bEOSs (**B**), spEOSs (**C**), and submucosal eosinophils (**D**) are shown for subjects in the mild and moderate-to-severe asthma groups stratified by airway mucosal CCL26-high versus airway mucosal CCL26-low status (*x-axis, top labels*). **P* < .05 for airway mucosal CCL26-high versus airway mucosal CCL26-low status within the mild or moderate-to-severe asthma populations.

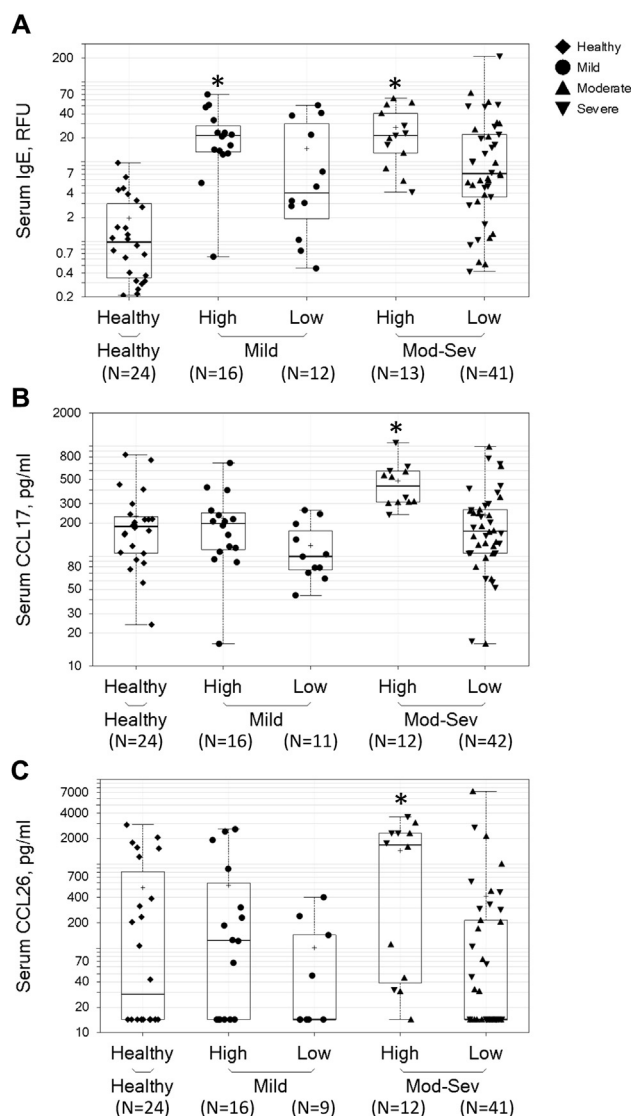


FIG 3. Type 2-associated serum biomarkers and airway mucosal CCL26 expression status. Baseline values of serum IgE (**A**), sCCL17 (**B**), and sCCL26 (**C**) are shown for subjects in the mild and moderate-to-severe asthma groups (*x-axis, bottom labels*) stratified by airway mucosal CCL26-high versus airway mucosal CCL26-low status (*x-axis, top labels*). * $P < .05$ for airway mucosal CCL26-high versus airway mucosal CCL26-low status within the mild or moderate-to-severe asthma populations. RFU, Relative fluorescence units.

between-group differences were less pronounced. For example, when defining airway mucosal type 2-high status based on CCL26, periostin, and IL-13-IVS expression, 100%, 80%, and 75% of patients with moderate-to-severe asthma with airway type 2-high status had high FENO values or high bEOS counts, but similar proportions of patients with moderate-to-severe asthma with airway type 2-low status had high FENO values or high bEOS counts (36%, 34%, and 32%), respectively. These observations reinforce our selection of airway mucosal CCL26 expression as the primary anchor for type 2 status.

Airway eosinophilia by airway CCL26 status

Thirty-nine patients with moderate-to-severe asthma had acceptable sputum quality, 8 of whom had high airway CCL26

expression, and comparisons were made with commonly used cutoffs, namely spEOS counts of 3% or greater and sputum neutrophil counts of 60% or greater (Fig 2, C, and Table IV). Most (88%) of the patients with high airway CCL26 expression but also 45% of those with low airway mucosal CCL26 expression had spEOS counts of 3% or greater ($P = .049$). Those with neutrophilic or paucigranulocytic patterns had predominantly low airway mucosal CCL26 expression (86% and 100%, respectively). Submucosal eosinophil intensity (histology) was available for 45 patients with moderate-to-severe asthma, 13 of whom had high airway mucosal CCL26 expression. Eosinophil intensity was significantly higher in patients with moderate-to-severe asthma with high airway mucosal CCL26 expression compared with those with low airway mucosal CCL26 expression ($P = .025$) and compared with healthy control subjects ($P = .0039$), with similar associations for mild asthma ($P = .024$ and $.0002$, respectively; Fig 2, D).

Airway mucosal gene expression by type-2-CCL26 status

Tables E4 and E5 (in this article's Online Repository at www.jacionline.org) present gene expression by CCL26-high or low status for mild, and moderate-severe asthma, respectively. Tables E6 and E7 present sputum inflammatory cells in the context of airway mucosal periostin and IL-13-IVS high or low status.

Classification of airway mucosal CCL26 type 2 status using clinical biomarkers

Prespecified thresholds for high/low FENO values, bEOS counts, and sCCL17 and sCCL26 expression were used to test classification of high airway CCL26 expression status (Table V; optimized thresholds from modeling are reported in Table E8 in this article's Online Repository at www.jacionline.org). Positive predictive values (PPVs) and negative predictive values (NPVs) were estimated based on the prevalences observed in the ADEPT study population. The actual predictive values for a given population were dependent on the prevalence of CCL26-high status in that population.

High FENO values, high bEOS counts, and the criterion of high FENO values OR high bEOS counts all had poor PPVs of 50% or less but good NPVs ($\geq 89\%$), with the high FENO value OR high bEOS count classification having the best NPV (100%). The criteria of high FENO value AND high bEOS count had only marginally better PPVs (55%) but with reduced NPVs (84%). High sCCL17 or high sCCL26 expression produced modestly improved PPVs (62% and 64%, respectively) while maintaining a good NPV (92% and 88%, respectively).

The combination of high FENO value OR high bEOS count AND high sCCL17 expression modestly improved PPVs (66%) while maintaining high NPVs (97%). The criteria high FENO value OR high bEOS count AND high sCCL26 expression resulted in much improved PPVs (88%) while maintaining good NPVs (89%). Finally, the combination of high sCCL17 expression AND high sCCL26 expression AND high FENO value OR high bEOS count criteria resulted in the best PPVs (100%) while maintaining good NPVs (87%). Therefore, depending on the application, one of these 3 combination models would be optimal, depending on whether a high NPV, a high PPV, or a balance of

TABLE IV. Comparison of airway mucosal type 2 CCL26 status and categorical biomarkers (high or low)

	Mild asthma			Moderate-to-severe asthma		
	Type 2 high (CCL26 ⁺), n = 16	Type 2 low (CCL26 ⁻), n = 12	P value*	Type 2 high (CCL26 ⁺), n = 13	Type 2 low (CCL26 ⁻), n = 42	P value*
FENO ≥35 ppb and blood eosinophils ≥300/mm ³	4 (25% [80%])	1 (8% [20%])	.36	6 (46% [55%])	5 (12% [45%])	.014
FENO ≥35 ppb or blood eosinophils ≥300/mm ³	12 (75% [75%])	4 (33% [25%])	.053	13 (100% [46%])	15 (36% [54%])	.000040
Neither FENO ≥35 ppb nor blood eosinophils ≥300/mm ³	4 (25% [33%])	8 (67% [67%])		0 (0% [0%])	27 (64% [100%])	
FENO ≥35 ppb	12 (75% [75%])	4 (33% [25%])	.053	9 (69% [47%])	10 (24% [53%])	.0063
FENO <35 ppb	4 (25% [33%])	8 (67% [67%])		4 (31% [11%])	31 (76% [89%])	
bEOS ≥300/mm ³	4 (25% [80%])	1 (8% [20%])	.36	10 (77% [50%])	10 (24% [50%])	.00090
bEOS <300/mm ³	12 (75% [52%])	11 (92% [48%])		3 (23% [9%])	32 (76% [91%])	
Serum IgE high	15 (94% [71%])	6 (50% [29%])	.023	12 (92% [29%])	29 (71% [71%])	.15
Serum IgE low	1 (6% [14%])	6 (50% [86%])		1 (8% [8%])	12 (29% [92%])	
Atopy (Phadiatop): positive	16 (100% [70%])	7 (64% [30%])	.019	11 (85% [26%])	31 (74% [74%])	.71
Atopy (Phadiatop): negative	0 (0% [0%])	4 (36% [100%])		2 (15% [15%])	11 (26% [85%])	
sCCL17 ≥75th percentile	3 (100% [100%])	0 (64% [0%])	.25	11 (85% [52%])	10 (74% [48%])	.00004
sCCL17 <75th percentile	13 (0% [54%])	11 (36% [46%])		1 (15% [3%])	32 (26% [97%])	
sCCL26 ≥75th percentile	4 (100% [100%])	0 (64% [0%])	.12	7 (85% [64%])	4 (74% [36%])	.0010
sCCL26 <75th percentile	12 (0% [52%])	11 (36% [48%])		5 (15% [12%])	38 (26% [88%])	
spEOS ≥3%	4 (33% [100%])	0 (0% [0%])	.25	7 (87.5% [33%])	14 (45% [67%])	.049
spEOS <3%	8 (67% [53%])	7 (100% [47%])		1 (12.5% [6%])	17 (55% [94%])	
Sputum neutrophils ≥65%	2 (33% [33%])	4 (0% [67%])	.13	3 (88% [25%])	9 (45% [75%])	.68
Sputum neutrophils <65%	10 (67% [77%])	3 (100% [23%])		5 (12% [19%])	22 (55% [81%])	
Paucigranulocytic sputum	6 (50% [67%])	3 (43% [33%])	.10	0 (0% [0%])	11 (35.5% [100%])	.15
Neutrophilic sputum	2 (17% [33%])	4 (57% [67%])		1 (12.5% [14%])	6 (19% [86%])	
Mixed granulocytic sputum	0 (0% [NA])	0 (0% [NA])		2 (25% [40%])	3 (10% [60%])	
Eosinophilic sputum	4 (33% [100%])	0 (0% [0%])		5 (62.5% [31%])	11 (35.5% [69%])	

Values are presented as numbers (percentages of type 2 group [percentages of row group]).

*P values (FDR) for comparison between airway CCL26 expression status (high/low) versus row variable subgroups (Fisher exact test or χ^2 test for spEOS-PMN subgroups).

Values are shown in boldface at an FDR of less than 0.05.

TABLE V. Airway mucosal CCL26 expression diagnostic characteristics of accessible biomarkers in patients with moderate-to-severe asthma

Airway mucosal CCL26-high classifiers*	Sensitivity	Specificity	AUC	PPV	NPV	Prevalence†
FENO ≥35 ppb OR bEOS ≥300/mm ³	100%	63%	0.82	46%	100%	24%
FENO ≥35ppb AND bEOS ≥300/mm ³	46%	88%	0.67	55%	84%	24%
FENO ≥35ppb	69%	76%	0.72	48%	89%	24%
bEOS ≥300/mm ³	77%	76%	0.77	50%	91%	24%
sCCL17 high	76%	85%	0.80	62%	92%	24%
(FENO ≥35 ppb OR bEOS ≥300/mm ³) AND sCCL17 high	93%	85%	0.89	66%	97%	24%
sCCL26 high	58%	90%	0.74	64%	88%	23%
(FENO ≥35 ppb OR bEOS ≥300/mm ³) AND sCCL26 high	58%	98%	0.77	88%	89%	23%
sCCL26 high AND sCCL17 high	50%	93%	0.71	67%	86%	23%
(FENO ≥35 ppb OR bEOS ≥300/mm ³) AND sCCL26 high AND sCCL17 high	50%	100%	0.75	100%	87%	23%

AUC, Area under the curve for receiver operating characteristic.

*Logistic regression model statistics for classification of patients with moderate-to-severe asthma to airway mucosal CCL26-high status.

†Prevalence based on the proportion of subjects with high airway mucosal CCL26 expression in the ADEPT moderate-to-severe asthma group, with PPV and NPV statistics based on this prevalence. The ADEPT study is a cross-sectional study with rigid enrollment criteria, and therefore the prevalence in other asthma study populations might vary based on recruitment criteria.

NPVs and PPVs is required. Fig E2 (available in this article's Online Repository at www.jacionline.org) presents the longitudinal stability of sCCL26 over 12 months. Fig E3 presents correlations between type-2 inflammation and eosinophilic phenotype biomarkers for mild (Fig E3, A), and moderate severe asthma (Fig E3, B) with sample sizes shown in Fig E4.

DISCUSSION

Based on the ADEPT study data set, we report that airway mucosal expression of CCL26 was a robust discriminator of type 2 inflammation from healthy nonatopic subjects. Furthermore,

airway mucosal CCL26 expression was best identified by using a panel of clinical biomarkers, including FENO values, bEOS counts, and expression of 2 novel markers, sCCL17 and sCCL26.

Airway mucosal CCL26 expression compared with periostin and IL-13-IVS expression provided the most robust segregation for airway mucosal type 2 activity status in our data set. The airway mucosal CCL26 type 2 phenotype also had the best concordance with airway eosinophilic inflammation, including 100% of patients with moderate-to-severe asthma and high airway mucosal CCL26 expression having high FENO values or high bEOS counts and 87.5% having high spEOS counts, compared with using periostin or IL-13-IVS as

anchors. Similar low proportions of patients with moderate-to-severe asthma with type 2–low status regardless of the airway mucosal type 2 anchor tested had high levels of eosinophilic inflammation. Taken together, these observations affirm our preference for airway mucosal CCL26 expression as the anchor for type 2 status. Similar findings pertain to the patients with mild asthma. Notwithstanding this preference, others have confirmed the substantial utility of periostin and other genes, including calcium-activated chloride channel regulator 1 (*CLCA1*) and serpin peptidase inhibitor, clade B, member 2 (*SERPINB2*; also known as plasminogen activator inhibitor 2), neither of which had quantifiable expression levels in the ADEPT study airway mucosa, as anchors for type 2 status,^{4,6} which are not inconsistent with the results presented in this report.

Both CCL26 and periostin expression are induced by IL-13 in multiple cell types present in endobronchial biopsy specimens, including epithelial cells, airway smooth muscle cells, and fibroblasts, as well as macrophages for CCL26, making them ideally suited for evaluation in biopsy specimens.^{11,23,24,32} CCL26 is a more effective chemoattractant for eosinophils than other chemokines, such as CCL11 and CCL24,³³ and in one report IL-13 induced greater expression of CCL26 in bronchial epithelial cells and higher supernatant CCL26 expression from patients with severe asthma compared with those with mild asthma, suggesting a role for CCL26 in the sustained inflammation seen in patients with severe eosinophilic asthma.²² The less consistent performance of periostin might be a consequence of TGF- β being a major regulator in addition to IL-4/IL-13.^{34,35} The lesser performance of IL-13–IVS might be related to the inclusion of genes not specific to IL-13 activity compared with specificity of CCL26 *per se*.

The majority of patients with mild but only a minority of those with moderate-to-severe asthma had high airway mucosal CCL26 expression, which was likely related to ICS treatment in the latter. Persistence of type 2–high status despite ICS treatment could be driven by steroid nonresponsiveness (or nonadherence), which is supported by the significant upregulation of the steroid-response gene *FKBP5* in patients with moderate-to-severe asthma with type 2–low, but not type 2–high, status (see Table E4). Consistent with this contention, increased CCL26 protein expression has been found in bronchial epithelium of patients with severe asthma selected for persistent high sEOS counts despite high corticosteroid doses.²²

In general, type 2 status was not associated with clinical asthma characteristics, perhaps related to the population with milder asthma in the ADEPT study, which had few patients with refractory asthma. In contrast, there were marked differences between those with high and those with low airway mucosal CCL26 expression for eosinophilia-associated clinical biomarkers, including FENO values, bEOS counts, and sEOS counts, which is consistent with the biology of CCL26, a potent eosinophil chemoattractant in asthmatic patients.³³ However, only about a third of sputum eosinophilic patients with moderate-to-severe asthma had high airway mucosal CCL26 expression (Table IV). This finding is consistent with a previous report by Choy et al³⁶ of only a fraction of sputum eosinophilic mild asthmatic patients having a T_H2 phenotype (evaluated based on periostin, *SERPINB2*, and *CLCA1* expression in endobronchial brushings). Type 2 activity, as evidenced by enriched IL-4/IL-13

downstream activity, would also be expected to coincide with IL-5 activity, which leads to eosinophil generation. This is consistent with asthmatic patients with high airway mucosal CCL26 expression having increased airway eosinophilic inflammation in both the airway submucosa and sputum. Of note, eosinophilia in blood, sputum, and the airway wall and FENO values might be discordant from each other, perhaps because of different mechanisms affecting these distinct compartments, such as IL-5 driving bEOS counts and IL-4/IL-13 increasing FENO values through local action on the airway mucosa³⁷ and bEOS counts and FENO values associating independently with wheezing episodes.³⁸ The presence of eosinophilia, as well as atopy/increased IgE levels with type 2–low status could reflect the transient nature of type 2–high status that was inhibited by ICSs or had spontaneously resolved. However, despite CCL26 being a potent inducer of airway eosinophilia,³³ recruitment of eosinophils to the airways could also occur independently of type 2 activity, such as from CCL11 (eotaxin-1) induced by non-type 2 mediators, such as TNF- α and IL-1 β .^{39–41}

sCCL17, an IL-13–induced chemoattractant for T_H2 cells,^{25,42} expression of which is reduced by anti-IL-13 and anti-IL-4 receptor therapeutics,^{10,13,26} was significantly higher in patients with moderate-to-severe asthma with high airway mucosal expression, despite ICS treatment. sCCL26 expression was also significantly increased in patients with moderate-to-severe asthma with high airway mucosal CCL26 expression. CCL26 could come from leakage from the airways, where transcriptional activity defines the airway mucosal CCL26–high phenotype. Larose et al²² showed a correlation between CCL26 expression and sEOS counts and suggested that bronchial epithelial cells are the dominant source of airway CCL26 that drives eosinophils into the airway lumen. Accordingly, CCL26 gene expression in endobronchial brushing samples from the ADEPT study was highly increased selectively in the group of asthmatic patients with high airway mucosal CCL26 expression (data not shown). In one report CCL26 gene expression in epithelial brushings was also highest in patients with severe asthma associated with higher sEOS counts, lower FEV₁, and more frequent exacerbations.⁴³

Knowing whether eosinophilic inflammation in the absence of type 2–high status is pathogenic will be important in determining which asthma subpopulations would benefit more from IL-4/IL-13– versus IL-5–targeted therapeutics. The efficacy of IL-4/IL-13–targeted therapeutics to affect eosinophilic inflammation is suggested by their capacity to reduce FENO values, as reported for lebrikizumab,¹³ although FENO value is not an absolute indicator of eosinophilic activity. The effect on sEOS counts by IL-4/IL-13–targeted therapeutics has not been reported. The efficacy of IL-5–targeted therapeutics in reducing type 2–associated pathology is unclear. Evidence of reductions in sIgE levels and CCL17 expression, as observed for the IL-4/IL-13–targeted therapeutics, would provide the clearest evidence for such but has not been reported.

Because the majority of effective anti-inflammatory agents work best in a type 2–high population, such as ICSs⁴ and anti-IL-13 antibodies,¹³ defining type 2 status in patients by using clinically accessible biomarkers will be important. Identification of subjects with high airway mucosal CCL26 expression among patients with moderate-to-severe asthma was best achieved based on combinations of high FENO value, high bEOS

count, high sCCL17 expression, and high sCCL26 expression criteria. Classification based on high FENO values OR high bEOS counts could be readily implemented in screening of asthmatic patients because these biomarkers can be routinely measured at present. Although having a perfect NPV for classifying high airway mucosal CCL26 status, the PPV (47%) was poor, limiting the utility of this classification for a codiagnostic test for the type 2–high phenotype. However, the classification could still be useful for enriching a study population for a type 2–low phenotype.

Adding high sCCL17 expression to the classification with high FENO values OR high bEOS counts maintained an optimal NPV (97%) while moderately improving the PPV to 66%. This classification has the potential to be implemented as a codiagnostic tool to triage patients likely to not benefit from type 2–targeting therapeutics while moderately enriching for those who would likely benefit.

Adding high sCCL26 expression to the classification with high FENO values OR high bEOS counts provided more of a balance between NPV (89%) and PPV (88%). The full model, including high FENO value OR high bEOS count, high sCCL17 expression, and high sCCL26 expression criteria had the best PPV (100%), with a modestly reduced NPV of 87%. These last 2 classification models might be useful to prospectively select patients for type 2–targeted therapeutic clinical studies, highly enriching for patients more likely to respond. However, these models have low sensitivities that would result in about half of the patients with type 2–high status being classified as having type 2–low status, which might be tolerable for proof-of-concept studies but not for application as a codiagnostic test.

These classification models will need to be confirmed in independent study populations, with ultimate validation coming from interventional clinical trials. The generalizability of our findings is unknown because the ADEPT study did not include smokers, subjects with high body mass index, and those requiring chronic oral corticosteroids. Because of the limited sample size of the type 2–high asthma subpopulation, training test set confirmation approaches could not be used. To limit risks of overmodeling, thresholds for categorization of the accessible biomarkers were predefined before modeling, although the optimal modeled thresholds performed similarly. In *post hoc* analyses logistic regression modeling with cross-validation (in the full study population without randomized training and test sets) resulted in an optimal model in which the weights for each categorical predictor (FENO or value bEOS count, sCCL17 expression, and sCCL26 expression) or continuous values for each predictor resulting in comparable model performance were compared with the simple Boolean models presented in Table V, with stronger performance by discrete predictors compared with the respective continuous values (see Table E9 in this article's Online Repository at www.jacionline.org). The ultimate cutoffs for the predictors would need to be retrospectively established from interventional clinical response data, with current thresholds providing for an enrichment to increase the probability of success for a therapeutic agent in the absence of available clinical response data. The stability of the airway mucosal type 2 phenotype over time has not been directly evaluated. The consistency of sCCL26 expression reported here suggests that the airway mucosal type 2 phenotype is a stable phenotype.

Limitations of this study include the absence of smoking, oral corticosteroid dependence, and morbidly obese asthmatic

subjects, as described previously.¹⁶ Furthermore, we did not recruit an atopic healthy subject population, which might have provided an additional group for comparison purposes. Finally, we did not have a reliable serum periostin assay available to enable comparison of expression of this gene with sCCL17 and sCCL26 measurements. However, as reported by Choy et al,³⁶ serum periostin expression was not significantly greater in patients with mild asthma with an airway mucosal T_H2 phenotype than in those with a non-T_H2 phenotype but rather increased only in those with an airway mucosal T_H17 phenotype.

In conclusion, airway mucosal CCL26 gene expression, representing airway mucosal IL-13 activity, was used to optimally define an airway mucosal type 2–high phenotype, which is a subgroup of a broader eosinophilic phenotype. Combinations of the clinically accessible biomarkers FENO, bEOS, sCCL17, and sCCL26 best identified the airway mucosal CCL26–high phenotype, and this promises to help select patients for type 2 inflammation–targeted therapies. Patients with type 2–low asthma, a group with high unmet need and few therapeutic options, can be reliably identified by using the same biomarkers.

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Key messages

- A type 2 inflammation phenotype has emerged as the target of novel asthma therapies.
- Airway mucosal gene expression of CCL26, periostin, or the multigene IL-13–IVS were used to segregate subjects with the type 2–high phenotype from those with the type 2–low phenotype. In our data set airway mucosal CCL26 expression was optimal.
- Combinations of clinical biomarkers, FENO values, bEOS counts, and sCCL17 and sCCL26 expression were optimal for identification of type 2 inflammation based on airway mucosal CCL26 expression, with similar findings for periostin and IL-13–IVS.

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