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ORIGINAL ARTICLE

Low CC16 mRNA Expression Levels in Bronchial Epithelial Cells Are Associated with Asthma Severity

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Abstract

Rationale: CC16 is a protein mainly produced by nonciliated bronchial epithelial cells (BECs) that participates in host defense. Reduced CC16 protein concentrations in BAL and serum are associated with asthma susceptibility.

Objectives: Few studies have investigated the relationship between CC16 and asthma progression, and none has focused on BECs. In this study, we sought to determine if CC16 mRNA expression levels in BECs are associated with asthma severity.

Methods: Association analyses between CC16 mRNA expression levels in BECs (242 asthmatics and 69 control subjects) and asthma-related phenotypes in Severe Asthma Research Program were performed using a generalized linear model.

Measurements and Main Results: Low CC16 mRNA expression levels in BECs were significantly associated with asthma susceptibility and asthma severity, high systemic corticosteroids use, high retrospective and prospective asthma exacerbations, and low pulmonary function. Low CC16 mRNA expression levels were significantly associated with high T2 inflammation biomarkers (fractional exhaled nitric oxide and sputum eosinophils). CC16 mRNA expression levels were negatively correlated with expression levels of Th2 genes (*IL1RL1*, *POSTN*, *SERPINB2*, *CLCA1*, *NOS2*, and *MUC5AC*) and positively correlated with expression levels of Th1 and inflammation genes (*IL12A* and *MUC5B*). A combination of two nontraditional T2 biomarkers (CC16 and IL-6) revealed four asthma endotypes with different characteristics of T2 inflammation, obesity, and asthma severity.

Conclusions: Our findings indicate that low CC16 mRNA expression levels in BECs are associated with asthma susceptibility, severity, and exacerbations, partially through immunomodulation of T2 inflammation. CC16 is a potential nontraditional T2 biomarker for asthma development and progression.

Keywords: asthma severity; asthma exacerbations; bronchial epithelial cells; CC16; T2 inflammation

CC16 (club cell secretory protein-16), encoded by the *SCGB1A1* gene on chr11q12.3, is predominantly produced by nonciliated bronchial epithelial cells (club cells) (1). CC16 may have antiinflammatory, immunomodulatory, antitoxicant, and antioxidative functions (2, 3). CC16 represents a biomarker for airway epithelium integrity or acute lung epithelium injury (4–7). In population-based studies, low serum CC16 protein concentrations have been associated with decreased lung function, exacerbations, and low pulmonary function. Low CC16 mRNA increased airway hyperresponsiveness (AHR), accelerated lung function decline, development of chronic obstructive pulmonary disease (COPD), and lung cancer-driven mortality (8–13). Smoking reduces the number of CC16-producing club cells and

CC16 protein concentrations in BAL and serum, and low CC16 protein concentrations are associated with a smoking-related decline in lung function (14–16). In patients with COPD (vs. smoking control subjects), low serum CC16 protein concentrations have been associated with decreased lung function, accelerated lung function decline, emphysema, COPD, and mortality (17–21).

In patients with asthma (vs. nonsmoking control subjects), low CC16 protein concentrations in BAL, serum, and urine have been associated with asthma susceptibility (22–26). Immunohistochemical staining in small airways has shown decreased numbers of CC16-producing club cells and increased numbers of T cells, activated eosinophils, and mast cells in patients with asthma compared with healthy control subjects (27). Previous studies did not find significant differences in CC16 protein concentrations in BAL with asthma severity, but they had small sample sizes (and even smaller numbers of patients with severe asthma) and did not directly address gene expression in BECs (22–23). In this study, we sought to determine if CC16 mRNA expression levels in BECs are associated with asthma severity in the well-characterized SARP (Severe Asthma Research Program) cohort.

Some of the results of these studies have been previously reported in the form of an abstract (28).

Methods

Study Participants
SARP is a currently active multicenter program funded for the last 20 years by the NHLBI (National Heart, Lung, and Blood Institute) involving nonsmokers with mild to severe asthma (enriched for severe asthma on the basis of the ATS–ERS [American Thoracic Society–European Respiratory Society] criteria [29]) and a subset of healthy control subjects. Participants in SARP were comprehensively phenotyped using standard protocols as described previously (30–40) and in the online supplement. Bronchoscopy was performed on a subset of participants (age >12 yr) in the SARP longitudinal cohort (n = 156) and cross-sectional cohort (n = 155) to obtain epithelial cells from brush biopsies for RNA sequencing (RNAseq) and microarray mRNA chip analysis, respectively (without overlapped participants). SARP was approved by the appropriate institutional review board, including informed consent.

Statistical Analysis
Association analysis of CC16 mRNA expression levels and asthma-related phenotypes. RNAseq and mRNA microarray data from bronchial epithelial cells (BECs) in the longitudinal and cross-sectional cohorts were extracted for the *SCGB1A1* gene and 16,067 and 19,566 other genes, respectively, as described previously (32, 41–43) and in the online supplement. The RNAseq and microarray expression data have been deposited and can be accessed through dbGaP (phs001446) and GEO (GSE63142 and GSE43696), respectively (32, 41, 43–45). Association analyses of CC16 mRNA expression levels and asthma-related phenotypes were performed using a logistic regression, a linear regression, and a negative binomial model for binary, continuous, and counting variables, respectively, adjusted for age, sex, body mass index (BMI), race, and batch effect using SAS 9.4 software (SAS Institute Inc.). Nominal P value of 0.05 was considered significant.

Association analysis of CC16–IL-6 and asthma-related phenotypes. To increase sample size and power, patients with asthma in the cross-sectional and longitudinal cohorts were merged on the basis of the standardized median value of CC16 mRNA expression levels. In brief, CC16 mRNA expression levels in patients with asthma in the cross-sectional cohort and the longitudinal cohort were standardized by dividing the median of CC16 mRNA expression levels of the nonsmoking healthy control subjects in the cross-sectional cohort (n = 27; median = 18.83) and the longitudinal cohort (n = 42; median = 16.33), respectively. The standardized median value of CC16 mRNA expression levels in patients with asthma in the cross-sectional cohort was used as a cut-off value (0.9684) to categorize patients with asthma into high CC16 (n = 108) and low CC16 (n = 134) groups in the SARP cross-sectional and longitudinal cohorts. Patients with asthma with CC16 mRNA expression levels were further categorized into high IL-6 (n = 50) and low IL-6 (n = 61) groups on the basis of the median value of blood IL-6 protein concentrations in patients with asthma (n = 763; cut-off value = 1.58 pg/ml) in the SARP cross-sectional and longitudinal cohorts (37). Association analyses of asthma-related phenotypes stratified by high and low CC16 or a combination of CC16 mRNA expression levels in BECs and blood IL-6 protein concentrations: HH (high CC16 and high IL-6; n = 19), HL (high CC16 and low IL-6; n = 28), LH (low CC16 and high IL-6; n = 31), and LL (low CC16 and low

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the Subject: Reduced CC16 protein concentrations in BAL and serum have been associated with asthma susceptibility, however, it has not been investigated in bronchial epithelial cells (BECs), the most relevant cell type in which CC16 is primarily produced. In addition, very few studies have investigated the relationship between CC16 and asthma progression.

What This Study Adds to the Field: This study shows that reduced CC16 mRNA expression levels in BECs are associated with asthma susceptibility and asthma severity. More specifically, among patients with asthma, reduced CC16 mRNA expression levels are associated with decreased lung function, enhanced systemic corticosteroids use, and increased asthma exacerbations. Importantly, reduced CC16 mRNA expression levels are associated with increased T2 biomarkers and increased expression levels of T2 pathway genes. Furthermore, a combination of two nontraditional T2 biomarkers (CC16 mRNA expression levels in BECs and blood IL-6 protein concentrations) reveals four distinct asthma endotypes with different characteristics of T2 inflammation, obesity, and asthma severity. In summary, low CC16 mRNA expression levels in BECs are associated with asthma progression, probably through upregulation of T2 inflammation. Thus, CC16 is a potential nontraditional T2 biomarker for asthma development and progression.

IL-6; $n = 33$) were performed using a nonparametric Kruskal-Wallis test and a generalized linear regression model adjusted for age, sex, BMI, race, and batch effect. Nominal P value of 0.05 was considered significant.

Meta-analysis of genes correlated with CC16 mRNA expression levels. Correlation analyses of CC16 mRNA expression levels in BECs with 16,067 and 19,566 genes in the longitudinal and cross-sectional cohorts, respectively, were performed using Spearman correlation as described previously (32). Meta-analysis of Spearman correlation coefficient (rho) was performed in the

overlapped 14,233 genes in the SARP cohorts ($n = 311$) using metacor R package (46). Bonferroni-adjusted P value of 3.513×10^{-6} (0.05/14,233) on the basis of the Olkin-Pratt fixed-effect meta-analytical model was considered significant.

Gene set enrichment analysis of genes significantly correlated with CC16 mRNA expression levels. A total of 801 and 670 genes were positively and negatively correlated with CC16 mRNA expression levels significantly on the basis of meta-analysis, respectively. These 1,471 genes and the *SCGB1A1* gene were input into Enrichr software package for gene set enrichment analysis (47). P value and q-value (Benjamini-Hochberg adjusted P value) of the top 10 terms were extracted from GO (Gene Ontology) Biological Process 2021, GO Cellular Component 2021, KEGG (Kyoto Encyclopedia of Genes and Genomes) 2021 Human, Jensen TISSUES, CellMarker Augmented 2021, and dbGAP libraries.

Results

Demographics of SARP Participants with mRNA Expression in BECs

Bronchoscopy was performed on a subset of SARP participants to obtain epithelial cells from brush biopsies for mRNA expression analysis (Table 1). In the longitudinal cohort, RNAseq in BECs was performed in 156 participants (42 healthy control subjects, 52 patients with nonsevere asthma, and 62 patients with severe asthma). In the cross-sectional cohort, microarray mRNA expression in BECs was performed in 155 participants (27 healthy control subjects, 78 patients with nonsevere asthma, and 50 patients with severe asthma). There were no overlapped participants with mRNA expression in BECs in the longitudinal and cross-sectional cohorts.

Demographics of the longitudinal and cross-sectional cohorts were similar (Table 1). Patients with severe asthma were older than patients with nonsevere asthma. Sex (percentage of females) and race (percentage of White, African American, and other) were not significantly different between healthy control subjects and patients with nonsevere or severe asthma. BMI showed an increasing trend, and baseline prebronchodilator (pre-BD) FEV$_1$% predicted, and FEV$_1$/FVC showed a decreasing trend from healthy

control subjects to nonsevere asthma, then to severe asthma.

Low CC16 mRNA Expression Levels in BECs were Associated with Asthma Susceptibility and Asthma Severity

CC16 mRNA expression levels in BECs were significantly higher in healthy control subjects than patients with asthma in the cross-sectional cohort (18.9 vs. 17.9 [log2 transformed] or $2 \times$ difference; $P < 0.0001$) (Table 2 and Figure 1A) and in the longitudinal cohort (16.3 vs. 15.4 [natural log transformed] or $2.5 \times$ difference; $P < 0.0001$) (Table 2 and Figure 1B). CC16 mRNA expression levels in BECs were significantly higher in patients with nonsevere asthma than patients with severe asthma in the cross-sectional cohort (18.2 vs. 17.5 or $1.6 \times$ difference; $P = 0.005$) (Table 2 and Figure 1A) and in the longitudinal cohort (15.6 vs. 15.1 or $1.6 \times$ difference; $P = 0.058$) (Table 2 and Figure 1B).

Low CC16 mRNA Expression Levels in BECs were Associated with Asthma Exacerbations and Low Lung Function

Reduced CC16 mRNA expression levels in BECs were significantly associated with decreased baseline pre-BD FEV$_1$% predicted and FEV$_1$/FVC in patients with asthma in the cross-sectional cohort ($R^2 = 0.11$; $P = 0.002$) (Table 3 and Figure 2A) and longitudinal cohort ($R^2 = 0.16$; $P = 0.0008$) (Table 3 and Figure 2B). CC16 mRNA expression levels in BECs were significantly higher in patients with asthma without exacerbations than with exacerbations in the last 12 months in the cross-sectional cohort (18.2 vs. 17.5 or $1.6 \times$ difference; $P = 0.01$) (Table 4 and Figure 2C) and longitudinal cohort (15.6 vs. 15.0 or $1.8 \times$ difference; $P = 0.007$) (Table 4). More importantly, reduced CC16 mRNA expression levels in BECs were significantly associated with an increased number of prospective exacerbations over 3 years of follow-up in the longitudinal cohort (risk ratio = 1.75; $P = 0.02$) (Table 4 and Figure 2D).

Low CC16 mRNA Expression Levels in BECs were Associated with High T2 Biomarkers

In the cross-sectional and longitudinal cohorts, reduced CC16 mRNA expression levels in BECs were significantly associated with increased fractional exhaled nitric oxide

Table 1. Demographics (Mean ± SD) of Participants with mRNA Expression Measured in Bronchial Epithelial Cells

SARP Cross-sectional Cohort Microarray mRNA ($n = 155$)	Nonsmoking Control Subjects ($n = 27$)	Nonsmoking Asthma* ($n = 128$)	Nonsevere Asthma[†] ($n = 78$)	Severe Asthma[†] ($n = 50$)	P Value[‡] (Asthma vs. Control Subjects)	P Value[‡] (Severe vs. Nonsevere)
Age at enrollment, yr	33 ± 13	37 ± 13	33 ± 12	44 ± 11	0.19	<0.0001
Sex, n (%) female	15 (56)	86 (67)	51 (65)	35 (70)	0.27	0.7
Race (% White/African American/other[§])	70/19/11	60/31/9	56/32/11	65/29/6	0.43	0.55
Body mass index, kg/m^2	26 ± 5.2	30 ± 6.9	29 ± 6.7	32 ± 7.0	0.0016	0.029
Baseline Pre-BD FEV$_1$% predicted[‖]	94 ± 9.1	72 ± 22.1	82 ± 17	56 ± 20	<0.0001	<0.0001
Baseline Pre-BD FEV$_1$/FVC[‖]	0.81 ± 0.04	0.70 ± 0.12	0.75 ± 0.09	0.61 ± 0.12	<0.0001	<0.0001

SARP Longitudinal Cohort RNAseq ($n = 156$)	Nonsmoking Control Subjects ($n = 42$)	Nonsmoking Asthma* ($n = 114$)	Nonsevere Asthma[†] ($n = 52$)	Severe Asthma[†] ($n = 62$)	P value[‡] (Asthma vs. Control Subjects)	P value[‡] (Severe vs. Nonsevere)
Age at enrollment, yr	41 ± 13	41 ± 13	37 ± 12	44 ± 13	0.92	0.0032
Sex, n (%) female	25 (60)	74 (65)	34 (65)	40 (65)	0.58	1
Race (% White/African American/other[§])	69/17/14	63/25/12	67/21/12	60/27/13	0.57	0.69
Body mass index, kg/m^2	28 ± 5.5	31 ± 8.7	30 ± 9.1	32 ± 8.3	0.033	0.025
Baseline Pre-BD FEV$_1$% predicted[‖]	99 ± 12	76 ± 19	85 ± 15	69 ± 20	<0.0001	<0.0001
Baseline Pre-BD FEV$_1$/FVC[‖]	0.81 ± 0.04	0.70 ± 0.08	0.73 ± 0.09	0.68 ± 0.10	<0.0001	0.015

Definition of abbreviations: Pre-BD = prebronchodilator; SARP = Severe Asthma Research Program.
*For the longitudinal cohort, nonsmokers were never-smokers or previous smokers with less than 5 to 10 pack-years of tobacco use, depending on age. For the cross-sectional cohort, nonsmokers were never-smokers or previous smokers with less than 5 pack-years of tobacco use.
[†]Asthma severity (nonsevere or severe asthma) was on the basis of American Thoracic Society–European Respiratory Society criteria (29).
[‡]P value was generated using the Kruskal-Wallis test.
[§]Other races include Hispanic, Asian, American Indian, and mixed.
[‖]Pre-BD pulmonary function was presented.

(F_{NO}) concentrations ($R^2 = 0.13$; $P = 0.0003$ and $R^2 = 0.063$; $P = 0.002$) (Table 5 and Figures 3A and 3D) and sputum percentage eosinophils ($R^2 = 0.032$; $P = 0.08$ [borderline significant] and $R^2 = 0.13$; $P = 0.0009$) (Table 5 and Figures 3C and 3F). In contrast, the association of CC16 mRNA expression levels in BECs with blood eosinophils or neutrophils and sputum neutrophils was not consistent in the longitudinal and cross-sectional cohorts (Table 5 and Figure 3B and 3E).

Low CC16 mRNA Expression Levels in BECs were Correlated with Higher mRNA Expression Levels of Th2 Genes

Spearman correlation analyses were performed between CC16 mRNA expression levels and 16,067 genes in the longitudinal cohort ($n = 156$) or 19,566 genes in the cross-sectional cohort ($n = 155$). Meta-analysis of Spearman correlation coefficient (rho) was then performed in the overlapping 14,233 genes in the SARP cohorts ($n = 311$) (46). With Bonferroni correction, 801 and 670 genes were positively and negatively

correlated with CC16 mRNA expression levels, respectively (Table E1 in the online supplement). Among a subset of candidate genes selected on the basis of biological relevance, CC16 mRNA expression levels in BECs were negatively correlated with mRNA expression levels of the Th2 pathway and inflammation genes (*IL1RL1, IL18R1, POSTN, SERPINB2, CLCA1, NOS2, MUC5AC,* and *PLA2G4A*) (Table 6). CC16 mRNA expression levels in BECs were positively correlated with mRNA expression levels of the Th1 pathway and inflammation genes (*IL12A, MUC5B, C3, TLR5,* and *CXCL6*).

Gene Set Enrichment Analysis of Genes Correlated with CC16 mRNA Expression Levels in BECs

A total of 1,471 genes significantly correlated with CC16 mRNA expression levels in BECs were input into Enrichr software package for gene set enrichment analysis (47). In the GO Biological Process 2021 library (Table E2 and Figure E1A), genes involved in the cytokine-mediated signaling pathway, negative

regulation of cell migration, epithelium development, and epithelial cell differentiation were enriched. In the GO Cellular Component 2021 library (Table E3 and Figure E1B), genes involved in the MHC class II protein complex were enriched. In the Jensen TISSUES library (Table E4 and Figure E1C), genes expressed in the lung and esophagus were enriched. In the CellMarker Augmented 2021 library (Table E5 and Figure E1D), genes expressed in secretory cells and type II pneumocytes in the lung were enriched. In the KEGG 2021 Human library (Table E6 and Figure E1E), genes associated with asthma and Th1/Th2 cell differentiation were enriched. In the dbGaP library (Table E7 and Figure E1F), genes associated with asthma were enriched.

Association of High–Low CC16 mRNA Expression Levels in BECs with Asthma Phenotypes

To increase sample size and statistical power, patients with asthma in the cross-sectional

Table 2. Association of CC16 mRNA Expression Levels in Bronchial Epithelial Cells with Asthma Susceptibility and Asthma Severity

SARP Cross-sectional Cohort Microarray mRNA (*n* = 155)	Nonsmoking Control Subjects (*n* = 27)	Nonsmoking Asthma* (*n* = 128)	OR (*P* Value)†	Nonsevere Asthma‡ (*n* = 78)	Severe Asthma‡ (*n* = 50)	OR (*P* Value)†
CC16 mRNA expression levels (log2)	18.9 ± 0.36	17.9 ± 1.14	0.10 (<0.0001)	18.2 ± 0.86	17.5 ± 1.36	0.55 (0.0049)

SARP Longitudinal Cohort RNAseq (*n* = 156)	Nonsmoking Control Subjects (*n* = 42)	Nonsmoking Asthma* (*n* = 114)	OR (*P* value)†	Nonsevere Asthma‡ (*n* = 52)	Severe Asthma‡ (*n* = 62)	OR (*P* value)†
CC16 mRNA expression levels (ln)	16.3 ± 0.44	15.4 ± 1.01	0.14 (<0.0001)	15.6 ± 0.97	15.1 ± 1.00	0.67 (0.058)

Definition of abbreviations: ln = natural logarithm; log2 = logarithm base 2; OR = odds ratio; RNAseq = RNA sequencing; SARP = Severe Asthma Research Program.
*For the longitudinal cohort, nonsmokers were never-smokers or previous smokers with less than 5 to 10 pack-years of tobacco use, depending on age. For the cross-sectional cohort, nonsmokers were never-smokers or previous smokers with less than 5 pack-years of tobacco use.
†OR and *P* value were generated using a logistic regression model adjusted for age, sex, body mass index, race, and batch effect.
‡Asthma severity (nonsevere or severe asthma) was on the basis of American Thoracic Society–European Respiratory Society criteria (29). CC16 mRNA expression levels were log2- and ln-transformed in the SARP cross-sectional cohort and longitudinal cohort, respectively.

and longitudinal cohorts were merged (*n* = 242) on the basis of the standardized median value of CC16 mRNA expression levels. High CC16 mRNA expression levels in BECs were significantly associated with younger age ($P = 0.0003$), a higher percentage non-Hispanic White ($P = 0.003$), and lower BMI ($P = 0.03$) (Table 7). Consistent with the findings of continuous CC16 mRNA expression levels (Table E8), low CC16 mRNA expression levels were significantly associated with decreased pulmonary function, higher T2 biomarkers, and a greater percentage of exacerbations and

ATS–ERS asthma severity (adjusted $P < 0.05$). In addition, low CC16 mRNA expression levels were significantly associated with greater airway reversibility (percentage change in FEV_1% predicted to albuterol: 25 vs. 16; adjusted $P = 0.004$), greater AHR (PC_{20} to methacholine: 0.58 vs. 1.5; adjusted $P = 0.02$), higher ACQ-6 score (asthma control questionnaire-6: 1.61 vs. 0.95; adjusted $P = 0.03$), and greater percentage of use of high-dose ICS (61% vs. 40%; adjusted $P = 0.04$) and oral or systemic corticosteroids (48% vs. 28%; adjusted $P = 0.02$).

Association of a Combination of CC16 mRNA Expression Levels in BECs and Blood IL-6 Protein Concentrations with Asthma Phenotypes

Participants with asthma were further categorized into four groups on the basis of a combination of two nontraditional T2 biomarkers (CC16 mRNA expression levels in BECs and blood IL-6 protein concentrations): HH (high CC16 and high IL-6), HL (high CC16 and low IL-6), LH (low CC16 and high IL-6), and LL (low CC16 and low IL-6) (Table 8 and Figure 4). Patients with asthma in two low CC16

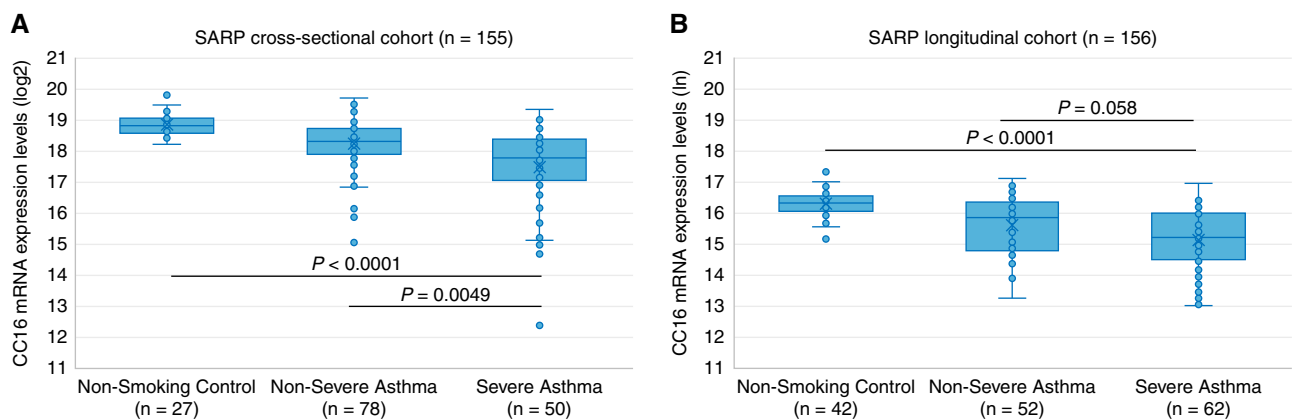**Figure 1.** Association of CC16 mRNA expression levels in bronchial epithelial cells with asthma susceptibility and asthma severity in the (*A*) SARP (Severe Asthma Research Program) cross-sectional cohort and (*B*) longitudinal cohort. CC16 mRNA expression levels have been logarithm base 2 (log2)- and natural logarithm (ln)-transformed in the (*A*) SARP cross-sectional cohort and (*B*) longitudinal cohort, respectively.

Table 3. Association of CC16 mRNA Expression Levels in Bronchial Epithelial Cells with Pulmonary Function in Asthmatics

SARP Cross-sectional Cohort Microarray mRNA (Asthmatics, $n = 128$)	Baseline Prebronchodilator FEV$_1$% Predicted ($n = 128$) β (P Value)*	Baseline Prebronchodilator FEV$_1$/FVC ($n = 127$) β (P Value)*
CC16 mRNA expression levels (log2)	4.99 (0.0021)	0.031 (0.0005)
SARP Longitudinal Cohort RNAseq (Asthmatics, $n = 114$)	**Baseline Prebronchodilator FEV$_1$% Predicted ($n = 114$) β (P value)***	**Baseline Prebronchodilator FEV$_1$/FVC ($n = 114$) β (P value)***
CC16 mRNA expression levels (ln)	5.69 (0.0008)	0.032 (0.0003)

Definition of abbreviations: ln = natural logarithm; log2 = logarithm base 2; RNAseq = RNA sequencing; SARP = Severe Asthma Research Program.
*Regression slope (β) and P value were generated using a linear regression model adjusted for age, sex, body mass index, race, and batch effect. CC16 mRNA expression levels were log2- and ln-transformed in the SARP cross-sectional cohort and longitudinal cohort, respectively.

groups (LH and LL) showed higher T2 biomarkers (median F$_{E_{NO}}$ ≥ 25 ppb), higher AHR, and lower pulmonary function. Patients with asthma in two high IL-6 groups (LH and HH) showed higher BMI (mean BMI ≥ 30 kg/m^2), higher blood neutrophils, and lower pulmonary function, similar to

what we have shown in a previous study (37). Patients with asthma in the HL group showed lower T2 biomarkers, lower AHR, and lower BMI. The HL group was a nonsevere asthma group (18% with severe asthma) with higher pulmonary function (baseline pre-BD FEV$_1$% predicted = 87),

lower ACQ-6 score (0.67), lower systemic corticosteroids use (21%), and lower asthma exacerbations (18%) in the last 12 months than the other three severe asthma groups (LH, LL, and HH), though not always statistically significant because of relatively small sample size.
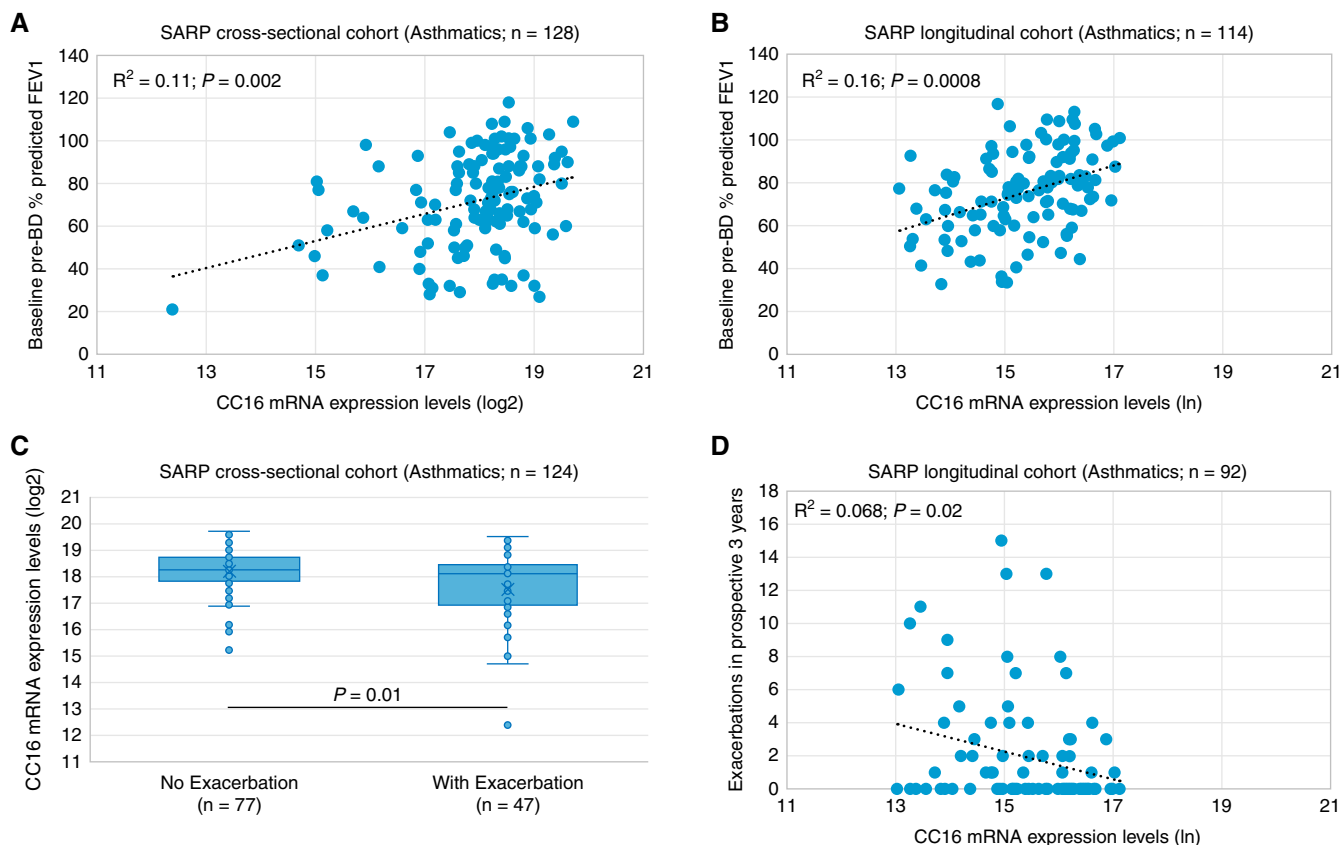
Figure 2. Association of CC16 mRNA expression levels in bronchial epithelial cells with (A and B) baseline FEV$_1$% predicted and (C and D) asthma exacerbations in the SARP (Severe Asthma Research Program) cross-sectional and longitudinal cohorts. CC16 mRNA expression levels have been logarithm base 2 (log2)- and natural logarithm (ln)-transformed in the (A and C) SARP cross-sectional cohort and (B and D) longitudinal cohort, respectively. Pre-BD = prebronchodilator.

Table 4. Association of CC16 mRNA Expression Levels in Bronchial Epithelial Cells with Asthma Exacerbations in Asthmatics

SARP Cross-sectional Cohort Microarray mRNA (Asthmatics, $n = 128$)	Exacerbation (Yes/No) in the Last 12 mo ($n = 124$)			Number of exacerbations in the prospective 3 yr ($n = 0$) RR (P Value)[†]
	No ($n = 77$)	Yes ($n = 47$)	OR (P Value)*	
CC16 mRNA expression levels (log2)	18.2 ± 0.83	17.5 ± 1.46	0.62 (0.012)	NA
SARP Longitudinal Cohort RNAseq (Asthmatics, $n = 114$)	**Exacerbation (Yes/No) in the Last 12 mo ($n = 114$)**			**Number of exacerbations in the prospective 3 yr ($n = 92$) RR (P Value)[†]**
	No ($n = 63$)	Yes ($n = 51$)	OR (P Value)*	
CC16 mRNA expression levels (ln)	15.6 ± 0.93	15.0 ± 1.02	0.55 (0.0067)	0.57 (0.015)

Definition of abbreviations: ln = natural logarithm; log2 = logarithm base 2; OR = odds ratio; RNAseq = RNA sequencing; RR = risk ratio; SARP = Severe Asthma Research Program.
*OR and P value were generated using a logistic regression model adjusted for age, sex, body mass index, race, and batch effect.
[†]RR and P value were generated using a negative binomial model adjusted for age, sex, body mass index, race, and batch effect. CC16 mRNA expression levels were logarithm base 2- and natural logarithm-transformed in the SARP cross-sectional cohort and longitudinal cohort, respectively.

Discussion

By using mRNA expression in BECs (the most relevant tissue for CC16 expression) in well-characterized SARP cross-sectional and longitudinal cohorts enriched for severe asthma, we have shown that low CC16 mRNA expression levels in BECs are associated with not only asthma susceptibility but also asthma severity. Our findings indicate that, among patients with asthma, reduced CC16 mRNA expression levels in BECs are associated with decreased lung function, enhanced systemic corticosteroids use, and increased retrospective and prospective asthma exacerbations. In addition, reduced CC16 mRNA expression levels are associated with increased T2 biomarkers and increased expression concentrations of Th2 pathway genes. We postulate that low CC16 expression levels in BECs upregulate airway T2 inflammation (Figure 5); chronic T2 inflammation in the airway may alter bronchial epithelium architecture and decrease the number of club cells, which further reduces CC16 expression levels in BECs.

Multiple factors may influence CC16 expression levels. In a population-based cohort, serum CC16 protein concentrations increased from birth to childhood and into the age of 32 years (48). In contrast, high CC16 mRNA expression levels in BECs were associated with younger age in adults with asthma (Tables 7 and E8). In a previous study, high BMI has been associated with low blood CC16 protein concentrations, indicating its potential role in obese asthma (49). In this study, low CC16 mRNA expression levels in BECs were also associated with high BMI in adults with asthma (Table 7). Smoking has shown strong effects on reducing CC16 protein concentrations (14–16), and thus, it should

Table 5. Association of CC16 mRNA Expression Levels in Bronchial Epithelial Cells with T2 AND non-T2 Biomarkers in Asthmatics

SARP Cross-sectional Cohort Microarray mRNA (Asthmatics, $n = 128$)	$F_{E_{NO}}$ ($n = 104$) β (P Value)*	Total Serum IgE ($n = 103$) β (P Value)*	Blood Eosinophil Counts ($n = 102$) β (P Value)*	Blood Neutrophil Counts ($n = 102$) β (P Value)*	Sputum % Eosinophils ($n = 62$) β (P Value)*	Sputum % Neutrophils ($n = 62$) β (P Value)*
CC16 mRNA expression levels (log2)	−0.10 (0.0003)	−0.088 (0.11)	0.11 (0.07)	−0.043 (0.017)	−0.20 (0.084)	−0.028 (0.43)
SARP Longitudinal Cohort RNAseq (Asthmatics, $n = 114$)	**$F_{E_{NO}}$ ($n = 113$) β (P Value)***	**Total Serum IgE ($n = 111$) β (P Value)***	**Blood Eosinophil Counts ($n = 114$) β (P Value)***	**Blood Neutrophil Counts ($n = 114$) β (P Value)***	**Sputum % Eosinophils ($n = 84$) β (P Value)***	**Sputum % Neutrophils ($n = 84$) β (P Value)***
CC16 mRNA expression levels (ln)	−0.10 (0.0022)	−0.080 (0.18)	−0.22 (0.0001)	−0.0009 (0.96)	−0.40 (0.0009)	0.069 (0.042)

Definition of abbreviations: $F_{E_{NO}}$ = fractional exhaled nitric oxide; ln = natural logarithm; log2 = logarithm base 2; RNAseq = RNA sequencing; SARP = Severe Asthma Research Program.
*Regression slope (β) and P value were generated using a linear regression model adjusted for age, sex, body mass index, race, and batch effect. CC16 mRNA expression levels were log2- and ln-transformed in the SARP cross-sectional cohort and longitudinal cohort, respectively.
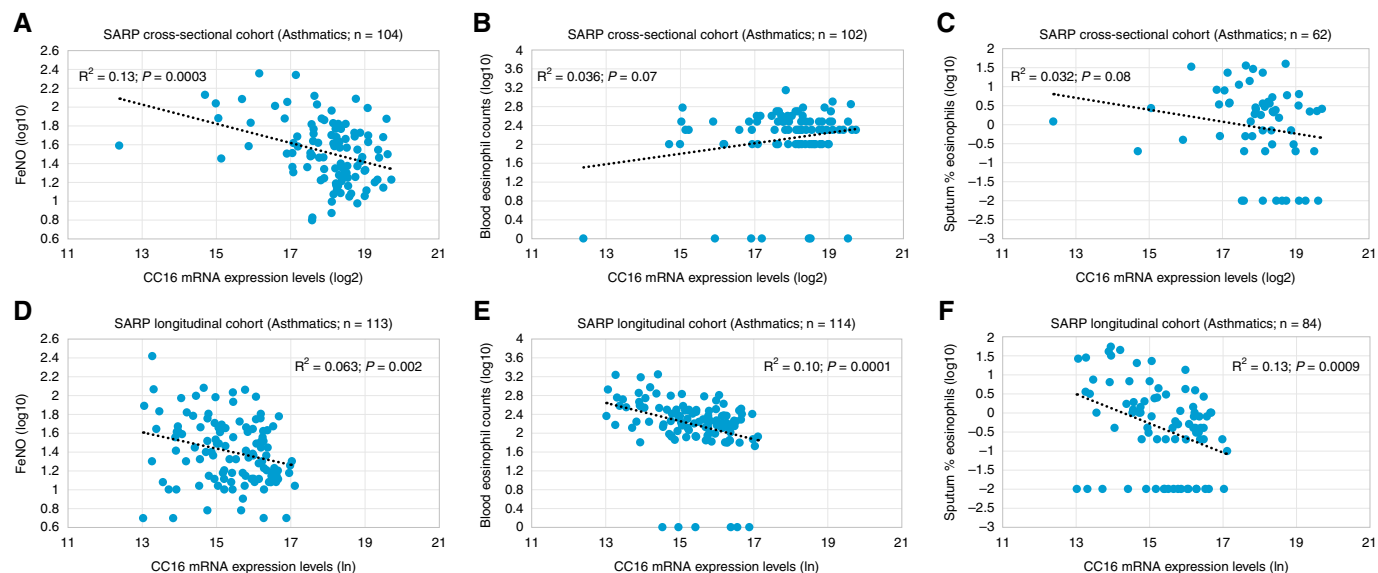
Figure 3. Association of CC16 mRNA expression levels in bronchial epithelial cells with (*A* and *D*) fractional exhaled nitric oxide ($F_{E_{NO}}$) values, (*B* and *E*) blood eosinophil counts, and (*C* and *F*) sputum percentage eosinophils in the SARP (Severe Asthma Research Program) cross-sectional and longitudinal cohorts. CC16 mRNA expression levels have been logarithm base 2 (log2)- and natural logarithm (ln)-transformed in the (*A–C*) SARP cross-sectional cohort and (*D–F*) longitudinal cohort, respectively.

be adjusted for when investigating CC16 in population-based or COPD cohorts (17–21). The SARP cohorts are "nonsmoking" asthma cohorts, including only a few former smokers with a low amount of tobacco use. In this study, smoking status (never smoker vs. former smoker) was not significantly associated with CC16 mRNA expression levels in BECs or asthma severity (Table E9). Adjustment for smoking status in addition to

age, sex, BMI, race, and batch effect showed no further effect on the significant association between CC16 mRNA expression levels in BECs and asthma severity.

It is unclear whether reduced CC16 expression levels upregulate expression of Th2 pathway genes, increased expression levels of Th2 pathway genes downregulate CC16 expression, or maybe both (Figure 5). An *in vivo* study using CC16-deficient mice

has shown increased expression of Th2 cytokines (IL-4/-5/-13) and eotaxin and heightened pulmonary eosinophilic inflammation (50). Reconstitution of the CC16 gene in CC16-deficient mice inhibited the expression of Th2 cytokines through inhibition of GATA-3 expression (51). An *in vitro* study using cord blood cells has found that CC16 inhibits Th2 cell differentiation of human naive neonatal

Table 6. Genes with Expression Levels Significantly Correlated with CC16 mRNA Expression Levels in Bronchial Epithelial Cells

Gene	SARP Cross-sectional Cohort ($n = 155$)		SARP Longitudinal Cohort ($n = 156$)		Meta-analysis of SARP Cohorts ($n = 311$)	
	rho*	*P* Value*	rho*	*P* Value*	rho[†]	*P* Value[†]
IL12A	0.34	2.02×10^{-5}	0.39	4.39×10^{-7}	0.37	5.55×10^{-14}
MUC5B	0.47	4.26×10^{-10}	0.47	6.05×10^{-10}	0.48	1.53×10^{-27}
TLR5	0.49	1.35×10^{-10}	0.54	$<2.20 \times 10^{-16}$	0.52	3.04×10^{-35}
C3	0.51	1.93×10^{-11}	0.48	2.31×10^{-10}	0.50	3.48×10^{-31}
CXCL6	0.44	9.26×10^{-9}	0.47	1.01×10^{-9}	0.46	2.10×10^{-24}
IL18R1	−0.37	1.66×10^{-6}	−0.32	4.38×10^{-5}	−0.35	1.37×10^{-12}
MUC5AC	−0.30	1.87×10^{-4}	−0.47	5.67×10^{-10}	−0.39	6.69×10^{-16}
PLA2G4A	−0.44	1.31×10^{-8}	−0.37	2.54×10^{-6}	−0.40	1.07×10^{-17}
POSTN	−0.41	1.09×10^{-7}	−0.32	4.00×10^{-5}	−0.37	3.28×10^{-14}
SERPINB2	−0.26	1.09×10^{-3}	−0.41	1.92×10^{-7}	−0.33	1.91×10^{-11}
CLCA1	−0.42	6.74×10^{-8}	−0.52	$<2.20 \times 10^{-16}$	−0.47	1.09×10^{-26}
IL1RL1	−0.39	6.36×10^{-7}	−0.44	1.07×10^{-8}	−0.42	4.55×10^{-19}
NOS2	−0.42	7.61×10^{-8}	−0.39	7.96×10^{-7}	−0.40	1.82×10^{-17}

Definition of abbreviation: SARP = Severe Asthma Research Program.
A subset of genes was presented in this table, whereas the full table was presented in Table E1.
*Correlation coefficient (rho) and *P* value were generated using a Spearman correlation model in which genes were inverse normalized with age, sex, body mass index, race, batch effect, and asthma status.
[†]Correlation coefficient (rho) and *P* value were generated on the basis of Olkin-Pratt fixed-effect meta-analytical model using metacor R package (46).

Table 7. Association of High–Low CC16 mRNA Expression Levels in Bronchial Epithelial Cells in Asthmatics with Asthma Phenotypes

SARP (Asthmatics, n = 242)	CC16 High* (n = 108)		CC16 Low* (n = 134)		P Value	
	n	Mean ± SD	n	Mean ± SD	Unadjusted[†]	Adjusted[‡]
Age at enrollment, yr	108	36 ± 13	134	42 ± 13	0.0003	NA
Age of asthma onset, yr	106	13 ± 13	132	16 ± 15	0.29	NA
Sex, n (%) female	108	76 (70)	134	84 (63)	0.22	NA
Race (% White/African American/other[§])	103	73/22/5	130	52/32/15	0.0026	NA
Body mass index, kg/m^2	108	29 ± 7.3	134	32 ± 8.1	0.025	NA
Baseline Pre-BD FEV$_1$% predicted[‖]	108	80 ± 20	134	69 ± 20	<0.0001	0.0005
Baseline Pre-BD FEV$_1$/FVC[‖]	108	0.74 ± 0.11	133	0.67 ± 0.11	<0.0001	<0.0001
Maximal FEV$_1$% predicted	96	91 ± 18	124	83 ± 20	0.0006	0.013
Maximal reversibility	108	16 ± 18	134	25 ± 25	0.0001	0.0042
PC$_{20}$ methacholine, mg/ml, median (IQR)	58	1.5 (0.5–3.1)	49	0.58 (0.28–2.0)	0.0053	0.016
Total serum IgE concentrations, IU/ml, median (IQR)	94	99 (29–307)	120	159 (86–336)	0.042	0.024
F$_{E_{NO}}$, ppb, median (IQR)	97	22 (15–42)	120	36 (19–63)	0.0024	0.0011
Blood eosinophils, cells/µl, median (IQR)	95	183 (100–300)	121	200 (100–374)	0.024	0.082
Blood neutrophils, cells/µl, median (IQR)	95	3,800 (2,900–5,008)	121	3,800 (2,906–5,548)	0.53	0.39
Sputum % eosinophils, median (IQR)	61	0.4 (0.001–2.2)	85	1.2 (0.2–4.4)	0.011	0.019
Sputum % neutrophils, median (IQR)	61	59 (33–78)	85	45 (30–63)	0.034	0.28
Serum or plasma IL-6 concentrations, pg/ml, median (IQR)	47	1.2 (0.68–2.3)	64	1.4 (0.74–3.0)	0.52	0.45
Serum sIL-6R concentrations, ng/ml, median (IQR)	49	37 (30–42)	73	37 (31–47)	0.21	0.10
High-dose ICS (%)	86	40	117	61	0.0043	0.040
Oral or systemic CS (%)	97	28	124	48	0.0035	0.015
Exacerbations in the last 12 mo, n (%)	106	33 (31)	132	65 (49)	0.0055	0.046
ACQ-6	42	0.95 ± 1.21	69	1.61 ± 1.40	0.0058	0.031
ATS-ERS severe asthma, n (%)	108	37 (34)	134	78 (58)	0.0003	0.0033

Definition of abbreviations: ACQ-6 = asthma control questionnaire-6; CS = corticosteroids; ATS-ERS = American Thoracic Society-European Respiratory Society; F$_{E_{NO}}$ = fractional exhaled nitric oxide; ICS = inhaled corticosteroids; Pre-BD = prebronchodilator; SARP = Severe Asthma Research Program.
*High and low CC16 mRNA expression levels in asthmatics were categorized using the standardized median value of CC16 mRNA expression levels as described in the Methods section.
[†]Unadjusted P value was generated using Kruskal-Wallis test.
[‡]Adjusted P value was generated using a generalized linear model adjusted for age, sex, body mass index, race, and batch effect.
[§]Other races include Hispanic, Asian, American Indian, and mixed.
[‖]Pre-BD pulmonary function was presented.

T cells by affecting dendritic cells (52). In this study, lower CC16 mRNA expression levels were associated with higher expression of Th2 pathway genes (*CLCA1, POSTN, SERPINB2, IL1RL1,* and *NOS2*) and inflammation genes (*PLA2G4A* and *PLA2R1*). Higher CC16 mRNA expression levels were associated with higher expression of the Th1 pathway gene (*IL12A*), complement pathway genes (*C3* and *C5*), and chemokines (*CXCL1, 2,* and *6*) involved in neutrophil chemoattractant and antimicrobial activity. A gene expression analysis in BECs has identified that mRNA expression of three T2 biomarkers (*CLCA1, POSTN,* and *SERPINB2*) is increased in patients with asthma, and expression of these genes can be upregulated by IL-13 or downregulated by corticosteroids treatment (53). The expression of the *NOS2* gene (nitric oxide synthase 2) in BECs is the main determinant for F$_{E_{NO}}$ concentrations (54). IL1RL1, the receptor for IL-33, is involved in

eosinophilic inflammation (55). In this study, CC16 mRNA expression levels are negatively associated with T2 inflammation biomarkers such as F$_{E_{NO}}$ and sputum eosinophils but are only weakly associated with total serum IgE concentrations (Table 5). Thus, it seems like CC16 can decrease F$_{E_{NO}}$ concentrations through downregulating IL-13 and NOS and decrease sputum eosinophils through downregulating IL-5 and IL1RL1, but less so for total serum IgE concentrations through IL-4.

It is unknown whether reduced CC16 expression levels cause asthma development or asthma leads to reduced CC16 expression levels (Figure 5). An early-life study with a relatively small sample size has shown that low plasma CC16 protein concentrations at 4 months of age do not predict the development of asthma at 3 years of age (56). Larger longitudinal population-based birth cohorts are needed to reveal the relationship between CC16 and asthma development.

rs3741240, a single-nucleotide polymorphism located at the 5′ untranslated region of the *SCGB1A1* gene, has been consistently associated with serum CC16 protein concentrations (57–58). In this study, the A allele of rs3741240 was also associated with low CC16 mRNA expression levels in BECs in non-Hispanic Whites in the SARP cross-sectional cohort (regression slope β = −0.81; P = 0.007) and longitudinal cohort (β = −0.69; P = 0.04) (Table E10). Several early candidate-gene association analyses with relatively small sample sizes have indicated that the A allele of rs3741240 was associated with asthma susceptibility (59–60); however, this finding was not confirmed by a meta-analysis (61). Although large-scale genome-wide association studies have not found significant associations between genetic variants in CC16 (including rs3741240) and asthma or other obstructive lung diseases (62), candidate gene studies have reported significant associations

Table 8. Association of a Combination of CC16 mRNA Expression Levels in Bronchial Epithelial Cells and Blood IL-6 Protein Concentrations in Asthmatics with Asthma Phenotypes

SARP (Asthmatics, n = 111)	HH (n = 19)		HL (n = 28)		LH (n = 31)		LL (n = 33)		P Value	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	Unadjusted*	Adjusted[†]
Age at enrollment, yr	19	41 ± 11	28	33 ± 11	31	44 ± 13	33	42 ± 11	0.004	NA
Age of asthma onset, yr	19	13 ± 12	28	12 ± 12	31	17 ± 15	33	16 ± 12	0.49	NA
Sex, n (%) female	19	12 (63)	28	20 (71)	31	26 (84)	33	13 (39)	0.002	NA
Race (% White/African American/other[‡])	19	53/47/0	28	89/11/0	31	42/42/16	33	48/33/18	0.002	NA
BMI, kg/m²	19	35 ± 8.1	28	27 ± 5.6	31	36 ± 10	33	27 ± 5.4	<0.0001	NA
Obesity (BMI ≥30 kg/m²), %	19	74	28	21	31	77	33	21	<0.0001	NA
Baseline Pre-BD FEV₁% predicted[§]	19	72 ± 19	28	87 ± 13	31	72 ± 17	33	67 ± 22	0.0002	0.029
Baseline Pre-BD FEV₁/FVC[§]	19	0.70 ± 0.09	28	0.75 ± 0.08	31	0.69 ± 0.08	33	0.64 ± 0.1	0.0004	0.0026
Maximal FEV₁% predicted	19	84 ± 16	28	96 ± 14	31	87 ± 15	33	83 ± 21	0.009	0.33
Maximal reversibility	19	20 ± 23	28	11 ± 9.0	31	24 ± 21	33	27 ± 26	0.002	0.042
PC₂₀ methacholine, mg/ml, median (IQR)	12	1.9 (0.49–3.2)	15	0.99 (0.39–2.4)	13	0.37 (0.17–0.71)	8	0.32 (0.18–0.60)	0.007	0.067
Total serum IgE concentrations, IU/ml, median (IQR)	19	140 (41–374)	27	139 (42–337)	30	168 (69–345)	32	153 (90–335)	0.97	0.99
FE_NO, ppb, median (IQR)	19	17 (14–33)	27	22 (14–44)	28	40 (27–66)	32	25 (13–46)	0.093	0.036
Blood eosinophils, cells/μl, median (IQR)	19	180 (68–252)	27	183 (100–300)	31	294 (100–400)	33	150 (100–304)	0.17	0.24
Blood neutrophils, cells/μl, median (IQR)	19	4,402 (3,825–5,280)	27	3,031 (2,300–4,095)	31	4,116 (2,777–5,969)	33	3,658 (3,088–5,044)	0.02	0.060
Sputum % eosinophils, median (IQR)	17	0.8 (0.3–2.6)	19	0.8 (0.1–3.2)	20	0.8 (0.2–7.9)	24	2.0 (0.5–4.0)	0.52	0.62
Sputum % neutrophils, median (IQR)	17	64 (33–88)	19	59 (48–76)	20	46 (32–76)	24	45 (30–69)	0.51	0.89
High-dose ICS (%)	15	47	22	36	29	48	31	52	0.74	0.89
Oral or systemic CS (%)	19	42	28	21	31	42	33	42	0.28	0.61
Exacerbations in the last 12 mo, n (%)	19	7 (37)	28	5 (18)	31	14 (45)	33	15 (45)	0.1	0.29
ACQ-6	11	0.91 ± 1.22	21	0.67 ± 0.80	19	1.68 ± 1.45	26	1.62 ± 1.47	0.022	0.13
ATS-ERS severe asthma, n (%)	19	7 (37)	28	5 (18)	31	14 (45)	33	16 (48)	0.07	0.55

Definition of abbreviations: ACQ-6 = asthma control questionnaire-6; BMI = body mass index; CS = corticosteroids; ATS-ERS = American Thoracic Society-European Respiratory Society; FE_NO = fractional exhaled nitric oxide; HH = high CC16 mRNA expression levels in bronchial epithelial cells and high blood IL-6 protein concentrations; HL = high CC16 and low IL-6; ICS = inhaled corticosteroids; LH = low CC16 and high IL-6; LL = low CC16 and low IL-6; Pre-BD = prebronchodilator; SARP = Severe Asthma Research Program.
*Unadjusted P value was generated using Kruskal-Wallis test.
[†]Adjusted P value was generated using a generalized linear model adjusted for age, sex, BMI, race, and batch effect.
[‡]Other races include Hispanic, Asian, American Indian, and mixed.
[§]Pre-BD pulmonary function was presented.

between rs3741240 and decline in lung function (63–64) and a recent Mendelian randomization study (58) has provided support toward a causal role of CC16 in COPD. Future studies will need to elucidate the nature of the association between low CC16 expression levels and asthma susceptibility.

It is also unknown whether reduced CC16 expression levels cause asthma progression or severe asthma leads to further reduced CC16 expression levels. GINA (Global Initiative for Asthma) guidelines and ATS–ERS define severe asthma as asthma that remains uncontrolled despite optimized treatment with high-dose ICS–LABA (inhaled corticosteroids-long-acting beta-agonists) or that requires high-dose ICS–LABA to prevent it from becoming uncontrolled (29, 65). The GINA definition

of severe asthma was derived from the ATS–ERS Task Force definition and represents the same definition of severe asthma. Thus, although GINA scores were not used in SARP, we would expect high GINA scores to also be associated with low CC16 mRNA expression levels in BECs. Because high-dose ICS and oral or systemic corticosteroids use is the major criterion to define severe asthma, it is difficult to dissect the influence of corticosteroids as a causal factor between CC16 mRNA expression levels and asthma severity in the current cross-sectional study design. As expected, high-dose ICS and oral or systemic corticosteroids use were significantly associated with both severe asthma and low CC16 mRNA expression levels in BECs (Table E11). Thus, adjustment for ICS doses in addition to age, sex, BMI, race, and batch

effect eliminated the significant association between CC16 mRNA expression levels in BECs and asthma severity, possibly because of overadjustment. Future studies will need to elucidate causality between low CC16 expression levels and asthma severity and the role of corticosteroids use in this relationship.

Biomarkers are biological indicators of a specific pathophysiological process, which can be used to stratify patients, track disease status and progression, and indicate drug effects. At the current stage, blood eosinophils, sputum eosinophils, FE_NO, and total serum IgE have been commonly used as biomarkers for T2 asthma (66). In contrast, biomarkers for non-T2 asthma are less well defined. Some potential nontraditional T2 asthma biomarkers are sputum neutrophils, Th1
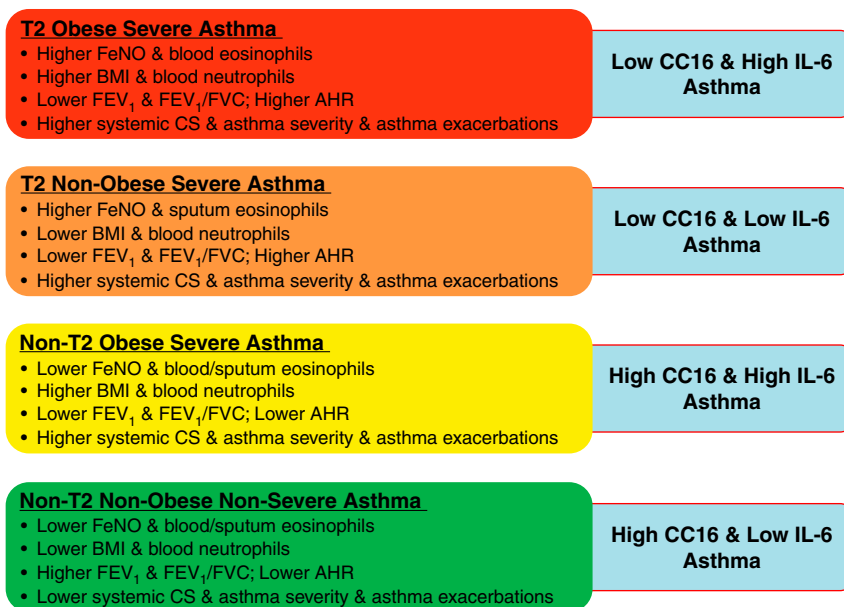
Figure 4. Summary of asthma endotypes defined by CC16 mRNA expression levels in bronchial epithelial cells and blood IL-6 protein concentrations. AHR = airway hyperresponsiveness; BMI = body mass index; CS = corticosteroids; $F_{E_{NO}}$ = fractional exhaled nitric oxide.

cytokines, Th17 cytokines, and inflammation biomarkers such as IL-6 (37, 66). Although most patients with asthma have evidence of persistent T2 endotypes, others have evidence that their persistent asthma is related to non-T2 mechanisms (30, 36, 37). Thus, the identification of nontraditional T2 asthma biomarkers is urgently needed. In this study, low CC16 mRNA expression levels are associated with asthma susceptibility, severity and exacerbations, low lung function, and high systemic corticosteroids use. In addition, CC16 mRNA expression levels are not significantly associated with the other two nontraditional T2 biomarkers (sputum neutrophils and blood IL-6 protein concentrations) (Table 7).

To increase sample size and power for investigating the combinatory effect of CC16 and IL-6, patients with asthma in the cross-sectional and longitudinal cohorts were merged. To accommodate the difference between the two mRNA measurement methods (microarray and RNAseq) (Figure 1), CC16 mRNA expression levels in patients with asthma in the cross-sectional and longitudinal cohorts were standardized by dividing the median of CC16 mRNA expression levels of the control subjects without asthma in the cross-sectional and the longitudinal cohorts, respectively. To accommodate the difference between the two SARP cohorts (different proportions of severe asthma) (Table 1), the standardized median value of CC16 mRNA expression

levels in patients with asthma in the cross-sectional cohort was used as a cut-off value to categorize patients with asthma into high CC16 and low CC16 groups in the cross-sectional and longitudinal cohorts. As for the high IL-6 and low IL-6 groups, the median value of blood IL-6 protein concentrations in all SARP patients with asthma was used as a cut-off value in the cross-sectional and longitudinal cohorts. In the future, potentially better cut-off values of CC16 and IL-6 could be investigated using sensitivity analysis in the large cohorts.

A combination of two nontraditional T2 biomarkers (CC16 and IL-6) reveals four asthma endotypes (i.e., LH, LL, HH, and HL groups, which represent T2 obese severe asthma, T2 nonobese severe asthma, non-T2
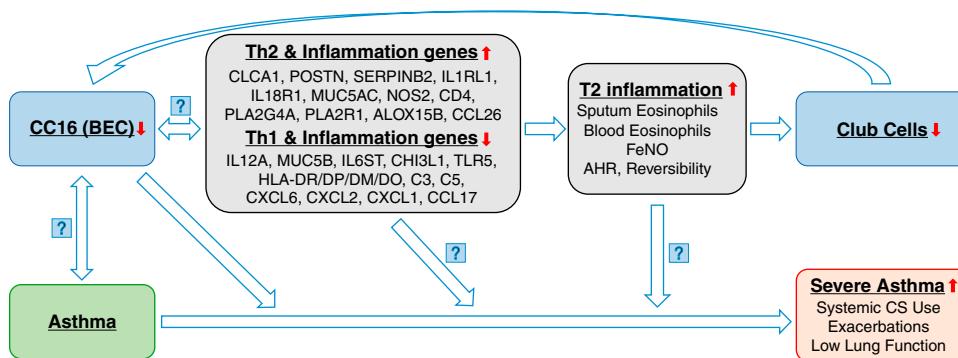

Figure 5. Asthma progression model with involvement of CC16 and T2 inflammation. AHR = airway hyperresponsiveness; BEC = bronchial epithelial cells; CS = corticosteroids; $F_{E_{NO}}$ = fractional exhaled nitric oxide.

obese severe asthma, and non-T2 nonobese nonsevere asthma, respectively) (Figure 4 and Table 8). In a previous analysis, we have shown that a combination of IL-6 (nontraditional T2 biomarker) and $F_{E_{NO}}$ (T2 biomarker) identified four asthma endotypes (37), including two severe asthma endotypes driven by high IL-6. In this study, a combination of CC16 and IL-6 identified four asthma endotypes, including three severe asthma endotypes driven by both low CC16 and high IL-6. The current analysis further shows the value of understanding two overlapped nontraditional T2 biomarkers and may represent a basis for targeted therapy in the future.

To further dissect CC16 as a nontraditional T2 asthma biomarker, the association of high–low CC16 mRNA expression levels in BECs with asthma severity was analyzed in atopic/nonatopic asthma (defined by positive/negative response to skin prick test in the cross-sectional cohort or specific IgE measurement in the longitudinal cohort) and in early/late onset asthma (age of asthma onset <12 or ≥12 yr) (Table E12). Asthmatics with low CC16 mRNA expression levels in BECs showed a consistently high percentage of severe asthma in atopic/nonatopic and early/late-onset asthma, which indicate that CC16 is a biomarker less affected by atopic status or age onset of asthma. Thus, CC16 is a consistent nontraditional T2 biomarker for asthma, asthma severity, and exacerbations.

Whether CC16 is truly a nontraditional T2 biomarker or simply a "negative" T2 biomarker is an important question. CC16 mRNA expression levels in BECs were associated with both T2 biomarkers (Tables 5 and E13) and selected gene expression biomarkers (Tables 6 and E14), but the magnitude of these correlations was moderate (0.25 < rho < 0.55) (67). T2 biomarkers ($F_{E_{NO}}$ and sputum eosinophils) (Table E13) as well as genes correlated with CC16 mRNA expression levels (Table 6) were not significantly associated with asthma severity, except for *PLA2G4A* in the longitudinal cohort (Table E14). In addition, the association between CC16 mRNA expression levels and asthma severity was not significantly affected by adjusting for T2 pathway genes (*POSTN*, *SERPINB2*, *CLCA1*, *IL1RL1*, and *NOS2*). Thus, CC16 should be considered a nontraditional T2 biomarker instead of a totally negative T2 biomarker.

There are some important questions that remain to be answered. Gene expression is dependent on the cell type or tissue, time and progression of asthma, and environmental factors, including exposures (32, 42, 43). It is critical that cells are obtained from the appropriate tissue (BECs for CC16) and from participants with the disease being investigated. BECs obtained from brush biopsies are mainly composed of epithelial cells (including ciliated cells, goblet cells, basal cells, and club cells), although there may be a small proportion of inflammatory and immune cells. A flow cytometry study showed that 95–97% of the cells from bronchial brushings were epithelial cells (68). In this study, cell populations were not available for every participant and, thus, were not adjusted. Future expression analyses by single-cell RNAseq of club cells may reveal interesting results. As a potential biomarker, the measurement of serum CC16 protein concentrations is more practical and noninvasive. However, the main source of serum CC16 is the respiratory epithelium because CC16 is a small secretory protein that can passively move across the airway–blood barrier into serum (3). Therefore, measurements of CC16 airway expression or CC16 concentrations in BAL or induced sputum may show stronger associations with asthma than serum CC16, as suggested by results in BAL from previous studies (23).

Conclusions

Our findings indicate low CC16 mRNA expression levels in BECs are associated with asthma susceptibility, severity and exacerbations, low lung function, and high systemic corticosteroids use. Although CC16 mRNA expression levels in BECs correlated with increased expression of Th2 genes and high T2 inflammation biomarkers, the association of CC16 with asthma severity was confirmed after adjustment for T2 pathway genes. Thus, CC16 is a potential nontraditional T2 biomarker for asthma development and progression. ∎

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