Club Cell Protein 16 and Disease Progression in Chronic Obstructive Pulmonary Disease

Hye Yun Park^{1,2}, Andrew Churg³, Joanne L. Wright³, Yuexin Li¹, Sheena Tam¹, S. F. Paul Man^{1,4}, Donald Tashkin⁵, Robert A. Wise⁶, John E. Connett⁷, and Don D. Sin^{1,4}

¹University of British Columbia James Hogg Research Center and the Institute for Heart and Lung Health, St. Paul's Hospital, Vancouver, British Columbia, Canada; ²Division of Pulmonary and Critical Care Medicine, Department of Medicine, Samsung Medical Center, Seoul, South Korea; ³Department of Pathology and ⁴Department of Medicine (Pulmonary Division), University of British Columbia, Vancouver, British Columbia, Canada; ⁵David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, California; ⁶Johns Hopkins University School of Medicine, Baltimore, Maryland; and ⁷University of Minnesota School of Public Health, Minneapolis, Minnesota

Rationale: Club (Clara) cell protein 16 (CC-16) is a protein that is synthesized predominantly in the lungs and is detectable in serum. Its expression decreases with lung injury and smoking, and is thus a marker of bronchial cell dysfunction.

Objectives: To evaluate the possibility of using serum CC-16 as a biomarker for disease progression in chronic obstructive pulmonary disease (COPD).

Methods: We measured serum CC-16 levels from 4,724 subjects with mild-to-moderate airflow limitation in the Lung Health Study. Using a linear regression model, we determined the relationship of serum CC-16 concentrations to decline in lung function over 9 years. In addition, to determine whether CC-16 plays a major role in the pathogenesis of mild COPD, we exposed CC-16-deficient (–/–) mice to 6 months of cigarette smoke.

Measurements and Main Results: Reduced serum concentrations of CC-16 were associated with accelerated decline in FEV₁ over 9 years ($P < 0.0001$), and this association persisted after adjustments for age, sex, race, smoking status, airway reactivity, body mass index, and baseline FEV₁ ($P = 0.0002$). However, CC-16^{–/–} mice did not demonstrate an enhanced risk of emphysema or small airway remodeling in response to cigarette smoke.

Conclusions: Serum CC-16 is associated with disease progression, and may assist in the identification of “rapid progressors.” However, the absence of CC-16 does not appear to modify the risk of cigarette-related COPD in mice.

Keywords: biomarker; chronic obstructive pulmonary disease; disease progression; smoking

Chronic obstructive pulmonary disease (COPD) is a condition characterized by progressive and rapid loss of lung function over time (1, 2). Although there are more than 200 million patients

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Club (Clara) cell protein 16 (CC-16) is a pneumoprotein that is synthesized predominantly in the lungs and found in serum. The expression of CC-16 has been reported to decrease with lung injury and smoking, and is thus a marker of bronchial cell dysfunction. However, the role of CC-16 as a biomarker for disease progression in chronic obstructive pulmonary disease (COPD) is not known.

What This Study Adds to the Field

This study shows that decreases in serum levels of CC-16 are significantly related to decline in FEV₁ over a span of 9 years with area under the curve values similar to those of bronchial hyperresponsiveness, which is a well known risk factor for rapid FEV₁ decline. Although the overall strength of the relationship between serum CC-16 and FEV₁ decline is relatively small, serum CC-16, in addition to clinical risk factors, may enable selection and enrichment of future COPD cohorts for “rapid” progressors and thus enhance the statistical power and precision of future interventional studies of COPD.

(Received in original form May 13, 2013; accepted in final form November 15, 2013)

Supported by Canadian Institutes of Health Research funding of the Lung Health Study (LHS) Biomarker Study; the original LHS was funded by the National Heart, Lung, and Blood Institute. D.D.S. is a Canada Research Chair in Chronic Obstructive Pulmonary Disease.

Author Contributions: All authors contributed to and approved the final draft of the manuscript; conception and design: D.D.S., S.F.P.M., A.C., and J.L.W.; experiment and data acquisition: A.C. and J.L.W. contributed to animal experiments, and D.D.S., Y.L., S.T., and H.Y.P. contributed to human sample experiments; analysis and interpretation: D.D.S., S.F.P.M., A.C., J.L.W., H.Y.P., D.T., R.A.W., and J.E.C.; drafting the manuscript: D.D.S. and H.Y.P.; critical revision of the manuscript: D.D.S., S.F.P.M., A.C., J.L.W., H.Y.P., Y.L., S.T., D.T., R.A.W., and J.E.C.

Correspondence and requests for reprints should be addressed to Don D. Sin, M.D., St. Paul's Hospital, 1081 Burrard Street, Vancouver, BC V6Z 1Y6, Canada. E-mail: don.sin@hli.ubc.ca

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

Am J Respir Crit Care Med Vol 188, Iss. 12, pp 1413–1419, Dec 15, 2013

Copyright © 2013 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201305-0892OC on November 18, 2013
Internet address: www.atsjournals.org

with COPD worldwide (3), there are no pharmacotherapies that can significantly modify lung function decline, and there are only a few promising drugs in the pipeline. One major impediment to drug discovery has been the absence of markers of disease activity that can identify patients who will experience rapid loss in lung function, except by performing repeated spirometry measurements in many patients over at least 3-year of follow-up. This is important, as COPD is a heterogeneous disorder and there are no accurate or reliable clinical features that can separate “rapid” progressors from “slow” progressors (4–7). Biomarkers of disease activity are thus urgently needed to address the growing worldwide burden of COPD, the global mortality rate of which will reach seven million in less than 10 years (8). The most promising biomarkers of disease activity are pneumoproteins (i.e., proteins synthesized predominantly in lungs). Club (Clara) cell protein-16 (CC-16) is one of these lung predominant molecules that have shown promise as a biomarker of disease activity. CC-16 is a 16-kD homodimeric protein secreted by nonciliated bronchiolar club cells, and is believed to have an anti-inflammatory property within the airways (9, 10). Recently, the ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints) investigators reported that serum CC-16 levels were reduced in patients with COPD compared with smokers without COPD (11), and were related to decline in lung function over 3 years (5). However, the effects were relatively modest, and the study lacked sufficient

statistical power to account for the confounding effects of smoking and age. The purpose of this study was twofold. First, we sought to confirm these findings over 9 years using the Lung Health Study (LHS) data and extend these findings by determining the role of serum CC-16 as a biomarker of disease activity in continued smokers, intermittent quitters and sustained quitters. Second, using mice deficient in CC-16 (CC-16^{-/-}), we sought to determine whether CC-16 plays a major role in the pathogenesis of mild COPD.

METHODS

Human Study

Subjects. We used data from the LHS cohort. The details of this cohort have been published previously (12). The original LHS study enrolled active smokers between the ages of 35 and 60 years who demonstrated mild to moderate airflow limitation on spirometry as defined by FEV₁ of less than 90%, but greater than or equal to 55% of predicted, in the presence of FEV₁/FVC ratio of less than 0.70 after bronchodilation. Individuals who had major diseases, such as cancer, which could have compromised follow-up, were excluded. After enrollment, the study participants were asked to visit the study center annually for 5 years. At these yearly visits, salivary cotinine levels and exhaled carbon monoxide concentrations were measured to verify smoking status of the participants. Participants were categorized as sustained quitters if they were biochemically validated nonsmokers at each annual visit for 5 years. Participants who were smokers at each annual visit were defined as continued smokers. Those whose smoking behavior varied were classified as intermittent quitters. In addition, 4,594 participants also underwent a methacholine provocation test at the baseline visit. The detailed method of these tests has been described previously (13). Airway reactivity was quantified by the O'Connor two-point slope method.

CC-16 measurements. At Year 5 of follow-up, the participants were asked for a blood sample, and approximately 89% of eligible participants consented. The blood samples were separated into their various components, transferred to the LHS data coordinating center on dry ice, and kept in -70°C freezers until use. The serum samples were thawed and CC-16 concentrations were measured using a commercially available ELISA kit (Biovendor, GmbH, Heidelberg, Germany) according to the manufacturer's instructions. The lower limit of detection of this assay was 0.650 ng/ml, and values that were below this level of detection were recorded as 0.325 ng/ml. The details are published elsewhere (14). The protocol of this study was approved by University of British Columbia/Providence Health Care Research Ethics Committee (no. H08-01864).

Lung function measurements. Spirometry was performed at the time of recruitment, annually for 5 years, and then approximately 11 years after recruitment. Owing to smoking cessation intervention and use of a bronchodilator (i.e., ipratropium), some individuals experienced a significant increase in FEV₁ during the first 2 years. In subsequent years, however, study participants (on average) experienced a linear rate of decline in FEV₁ (15, 16). To remove this "first 2 year" effect, we determined the rate of decline using Year 2 FEV₁ as the baseline measurement. In method 1, we calculated the rate of decline in lung function by taking the difference in FEV₁ between Year 2 (baseline) and Year 5, between Year 5 and Year 11, and between baseline and Year 11, and dividing these values by the elapsed time between the two measurements. In method 2, we determined the rate of decline in lung function by taking into account all of the measured spirometric data from Year 2 onwards for each of the study participant using a repeated measures modeling with PROC Mixed in SAS (SAS Institute, Cary, NC). As the results from this more complex model were similar to those of the simpler linear regression model, for parsimony, the primary analysis is based on method 1.

Vital status. At the Year-5 visit, the participants were also asked to consent to additional follow-up (LHS 3). Participants were followed for an additional 7–8 years, during which time their vital status data were captured biannually. Vital status was successfully determined for 98.3% of the participants.

Animal Study

To determine whether CC-16 could modulate cigarette-induced COPD changes in both airways and parenchyma, we obtained CC-16^{-/-} mice

on a C57BL/6J genetic background from Jackson Laboratories (Bar Harbor, ME). CC-16 deficiency was confirmed by genotyping. Beginning at age 3 months, groups of CC16^{-/-} ($n = 7$) or wild-type mice ($n = 7$) were exposed to whole smoke by consuming three Kentucky 2R1 cigarettes per day for 5 days per week over 6 months. Five air-exposed mice in each group served as controls. Mice were killed immediately after the last cigarette exposure.

Lung morphology. Lungs were harvested at the time mice were killed, inflated with low-melting point agarose at a pressure of 30 cm H₂O, and fixed in formalin for 48 hours. They were then sectioned in a sagittal plane. A random slice was submitted for paraffin embedding and sectioning and staining by hematoxylin and eosin for measurement of airspace size, or picosirius red for measurements of airways. To measure mean airspace size, we used a standard morphometric grid, with a total line length of 1.02 mm at $\times 200$ magnification, and 42 points. A total of 20 random sites was counted for each section, and the mean linear intercept was determined from the summed values of all sites. Using Image-Pro (Media Cybernetics, Bethesda, MD), the mean linear intercept and surface-to-volume ratio were determined.

Small airway morphometry. All rounded noncartilaginous airways (bronchioles) were identified and photographed. Using Image-Pro, we measured internal and external bronchiolar diameters. Wall area was measured and collagen content was determined using image segmentation. The data were normalized to the perimeter of the basement membrane. The mean value for all airways in each animal was determined and used for statistical analysis.

Immunohistochemical staining for neutrophils and macrophages. Details for each of the methods are described in the online supplement.

Statistical Analysis

To evaluate the relationship of serum CC-16 to (1) FEV₁ decline, (2) total mortality, and (3) disease-specific causes of mortality, we first divided the cohort into quintiles of CC-16. The baseline characteristics across the quintiles were compared using ANOVA for continuous variables and a Chi-square test for dichotomous variables. We used linear regression analysis to account for the possible confounding effects of age, sex, smoking status, airway reactivity, body mass index (BMI), baseline (Year 2) FEV₁, and race on the relative rate of decline in FEV₁, expressed in milliliters per year. Serum CC-16 was considered both as a continuous variable and as quintiles. For the latter, quintile 5 (i.e., the group with the highest CC-16 values) was the referent. A logistic regression model was used to assess the relationship of CC-16 quintiles to mortality endpoints using quintile 1 (i.e., the group with the lowest CC-16 values) as the referent. Statistical adjustments were made for all covariates indicated previously, and results are described in the online supplement (see Table E1 in the online supplement). We also determined the discriminatory property of serum CC-16 in predicting rapid progression of FEV₁ (defined as ≥ 40 ml/yr) (5) by constructing a receiver operator characteristic (ROC) curve and calculating the area under the curve (AUC) or the C statistic. To create the best model, we first conducted univariate analyses of the baseline factors and chose the variable with the highest AUC on the univariate analysis as the baseline covariate. To this baseline covariate, we added the variables with the second-highest AUC and then the variable with the third-highest AUC score, and so forth, until all the variables were exhausted. To the penultimate model, we added serum CC-16 to evaluate the incremental effects of serum CC-16 on AUC. We also used different cut-off values to define rapid decline of FEV₁ based on the Year 2 to Year 11 measurements (≥ 20 ml/yr, ≥ 40 ml/yr, ≥ 60 ml/yr, or ≥ 80 ml/yr). The arithmetic means of morphometric parameters and inflammatory cells between two groups in mice were compared using ANOVA with a Tukey's *post hoc* analysis. All analyses were performed using IBM SPSS Statistics 21.0 (IBM, Chicago, IL) and SAS, version 9.2. A two-sided P value less than 0.05 was considered significant.

RESULTS

Human Study

Clinical characteristics of the cohort. Serum CC-16 was measured in 4,724 LHS participants. Their demographic and clinical characteristics across quintiles of serum CC-16 concentrations

are presented in Table 1. FEV₁ (L) at baseline (Year 2), blood draw (Year 5), and Year 11 were positively related to serum CC-16 levels, whereas airway reactivity was inversely related to serum CC-16 levels. Continued smokers had the lowest serum CC-16 levels (3.10 ± 2.23 ng/ml; $P < 0.0001$ vs. sustained quitters), whereas sustained quitters had the highest level (4.35 ± 2.72 ng/ml) and intermittent quitters had moderate levels (3.90 ± 2.43 ng/ml; $P < 0.0001$ vs. sustained quitters) (*see* Figure 1). However, there was substantial overlap in serum CC-16 levels across smoking status.

Serum CC-16 and lung function decline. There was a significant inverse correlation between serum CC-16 levels in the natural logarithmic scale and the rate of decline in FEV₁ (ml/yr) ($r^2=0.0043$; $P = 0.0001$ and $P = 0.0006$ using Proc Mixed) (*see* Figure 2). As shown in Table 2, regardless of how FEV₁ decline was calculated, it accelerated along the serum CC-16 quintile gradient from Year 2 to Year 11 (ml/yr and % of baseline FEV₁; all $P < 0.0001$), from Year 2 to Year 5 ($P = 0.0014$ and $P < 0.0001$, respectively), and from Year 5 to Year 11 ($P = 0.0012$ and $P < 0.0001$, respectively). Even after adjustments for age, sex, race, baseline FEV₁, BMI, airway reactivity, and smoking status, serum CC-16 levels were still inversely related to rate of decline in FEV₁ (ml/yr). According to smoking status, only intermittent quitters had a significant correlation between the serum CC-16 levels and rate decline in FEV₁ (ml/yr) after adjustments for all relevant covariates ($P < 0.0001$) (*see* Figure E1).

Among the clinical variables, the most predictive for rapid decline of FEV₁ (defined as ≥ 40 ml/yr) (5) was smoking status, which had an AUC of 0.63 (95% confidence interval [CI] = 0.61–0.65). The next predictive variable was airway reactivity (AUC = 0.56; 95% CI = 0.54–0.58), followed by baseline FEV₁ and log CC-16 (AUC = 0.55; 95% CI = 0.53–0.57 for each of them), sex (AUC = 0.54; 95% CI = 0.52–0.56), age (AUC = 0.51; 95% CI = 0.49–0.53), BMI (AUC = 0.51; 95% CI = 0.49–0.53), and race (AUC = 0.50; 95% CI = 0.49–0.51) (*see* Table E2). The clinical variables (smoking, airway reactivity, baseline FEV₁, sex, age, BMI, and race) collectively had an AUC of 0.66 (95% CI = 0.65–0.68). The addition of serum CC-16 significantly increased the AUC of this model to 0.67 ($P = 0.0335$), thus

providing additional discrimination for rapid decline in FEV₁ beyond these clinical variables (*see* Table 3). To determine the “best” cutoff value to discriminate those with and without rapid decline of lung function (defined as ≥ 40 ml/yr) (5), a series of ROC curves at various CC-16 values was constructed after adjustments of age, sex, BMI, race, smoking status, airway reactivity, and baseline FEV₁. The highest ROC statistics were observed at a CC-16 cut-off value of 3.5 ng/ml (AUC = 0.67). In a multivariate analysis, this threshold value of CC-16 (< 3.5 ng/ml) was independently associated with rapid decline of FEV₁ (defined as ≥ 40 ml/yr) after adjustments for clinical variables (odds ratio = 1.358; 95% CI = 1.164–1.584; $P = 0.0001$).

Next, we determined the effect of using different cut-off values to define rapid decline of FEV₁ by calculating AUCs of models created from clinical variables (age, sex, BMI, race, smoking status, airway reactivity, and baseline FEV₁) with and without CC-16. The addition of serum CC-16 increased AUC significantly when cut-off values of 20 and 40 ml/yr were employed (AUC = 0.66, $P = 0.0006$ in 20 ml/yr and AUC = 0.67, $P = 0.0335$ in 40 ml/yr; Table E3).

Animal Study

Morphometric measures in wild-type and CC-16 knockout mice. As shown in Figures 3A–3C, mean airspace size (mean linear intercept; a measure of emphysema) and bronchiolar wall thickness, as well as bronchiolar wall area/unit basement membrane (both measures of small airway remodeling) were significantly increased in the smoke-exposed group of wild-type mice compared with the control group of wild-type mice. In the smoke-exposed CC-16^{-/-} mouse group, mean airspace size was also significantly higher than for those in the control group of CC-16^{-/-} mice, whereas bronchiolar wall thickness and wall area/unit basement membrane were not significantly different between the control group of CC-16^{-/-} mice and the smoke-exposed CC-16^{-/-} mouse group. When these morphometric measures were compared between the wild-type smoke-exposed and CC-16^{-/-} smoke-exposed groups, none of the parameters showed a significant difference.

Inflammatory cell infiltration in wild-type and CC-16 knockout mice. Figure 4 shows macrophage (Figure 4A) and neutrophil

TABLE 1. THE BASELINE CHARACTERISTICS OF THE LUNG HEALTH STUDY PARTICIPANTS ACCORDING TO QUINTILES OF CLUB CELL PROTEIN 16 LEVELS IN SERUM AT YEAR 5

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P Value for Trend
<i>n</i>	945	946	944	945	944	
CC-16, ng/ml	0.88 ± 0.49	2.21 ± 0.29	3.19 ± 0.29	4.38 ± 0.40	7.09 ± 2.58	<0.0001
Age, years	52.1 ± 6.4	$53.2 \pm 6.8^*$	$53.4 \pm 6.7^*$	$53.7 \pm 6.8^*$	$54.9 \pm 7.0^{*†‡}$	<0.0001
Men, %	43.7	58.1*	66.1*†	70.2*†	77.8*†‡	<0.0001
White, %	95.0	95.5	96.9	97.4	97.4	0.0006
BMI, kg/m ²	25.2 ± 4.1	25.6 ± 4.1	25.6 ± 3.9	25.5 ± 3.8	$25.8 \pm 3.6^*$	0.0041
Smoking status						<0.0001
Sustained quitters, %	6.0	14.8	19.5	23.1	25.2	
Intermittent quitters, %	21.0	25.8	29.6	30.1	34.7	
Continued smokers, %	73.0	59.4	51.0	46.9	40.0	
Pack-years of smoking	39.5 ± 17.9	39.9 ± 18.8	39.8 ± 18.6	39.6 ± 18.2	41.8 ± 19.7	0.0259
FEV ₁ , L						
At baseline, Year 2	2.5 ± 0.7	$2.6 \pm 0.6^*$	$2.8 \pm 0.6^{*†}$	$2.8 \pm 0.6^{*†}$	$2.9 \pm 0.6^{*†‡}$	<0.0001
At blood draw, Year 5	2.3 ± 0.7	$2.4 \pm 0.7^*$	$2.6 \pm 0.6^{*†}$	$2.7 \pm 0.6^{*†}$	$2.7 \pm 0.7^{*†‡}$	<0.0001
At Year 11	2.0 ± 0.7	$2.1 \pm 0.7^*$	$2.3 \pm 0.7^{*†}$	$2.4 \pm 0.7^{*†}$	$2.4 \pm 0.7^{*†‡}$	<0.0001
Airway reactivity	2.30 ± 0.95	2.19 ± 0.93	$2.12 \pm 1.0^*$	$2.01 \pm 0.94^{*†}$	$1.99 \pm 0.96^{*†‡}$	<0.0001

Definition of abbreviations: BMI = body mass index; CC-16 = Club (Clara) cell protein-16.

Airway reactivity was derived from the following formula: $\ln(2.105 - \text{two-point slope})$. Study participants were divided into five identical groups based on CC-16 levels. Data are presented as mean $\pm$ SEM ($n = 4,724$).

* $P < 0.05$ versus quintile 1.

† $P < 0.05$ versus quintile 2.

‡ $P < 0.05$ versus quintile 3.

§ $P < 0.05$ versus quintile 4.

Figure 1. Club cell protein 16 (CC-16) levels (ng/ml) according to smoking status.

(Figure 4B) cell counts per five high-power fields. Smoke exposure increased macrophage numbers in both the wild-type and CC-16^{-/-} mouse groups, and neutrophil numbers in the CC-16^{-/-} mouse group. However, there were no differences in the inflammatory cell numbers between wild-type smoke-exposed and CC-16^{-/-} smoke-exposed groups.

DISCUSSION

The most important finding of the present study was that serum CC-16 was inversely associated with accelerated rate of decline of FEV₁ over 9 years, but the magnitude of the effect was relatively small. Our data are consistent with those of the ECLIPSE investigators, who demonstrated a significant relationship of

serum CC-16 levels to the rate of decline in FEV₁ in patients with moderate to severe COPD (5). We extend these findings by showing that serum CC-16 levels are predictive of accelerated lung function decline (defined as ≥ 40 ml/yr of FEV₁) over 9 years of follow-up in those with mild to moderate COPD. Interestingly, we found that serum CC-16 was most predictive (for decline in lung function) in intermittent quitters. However, these data should be interpreted with caution, owing to multiple comparisons and lack of sufficient statistical power in other smoking groups, which may have precluded a significant signal.

In addition, similar to the ECLIPSE study, we found that serum CC-16 levels were reduced in continued smokers and increased with advancing age and among male subjects (9, 11, 17, 18). We also found that serum CC-16 levels increased with

Figure 2. The relationship between log-transformed serum club cell protein 16 (CC-16) levels (ng/ml) and rate of decline in FEV₁ (ml/yr) over 9 years in the Lung Health Study. The solid line represents mean rate of decline in FEV₁ (ml/yr) and the dotted lines represent the 95% confidence intervals. $r^2 = 0.0043$; $P = 0.0001$.

TABLE 2. THE RATE OF DECLINE IN FEV₁ ACCORDING TO SERUM CLUB CELL PROTEIN 16 LEVELS OVER 9 YEARS IN THE LUNG HEALTH STUDY

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P Value for Trend
Decline in FEV ₁ from baseline (Year 2) to Year 11						
ml/yr	59 ± 33	55 ± 36	53 ± 35	53 ± 35	52 ± 38	<0.0001
% of baseline FEV ₁	22.4 ± 12.9	19.5 ± 12.9	17.9 ± 11.9	17.5 ± 11.9	16.9 ± 12.7	<0.0001
Adjusted relative rate of decline, ml/yr (mean ± SE)	7.03 ± 1.95	3.02 ± 1.82	1.34 ± 1.78	0.88 ± 1.77	1 (Reference)	0.0002* (0.0233 [†])
Decline in FEV ₁ from baseline (Year 2) to Year 5						
ml/yr	67 ± 68	60 ± 65	57 ± 65	58 ± 69	56 ± 70	0.0014
% of baseline FEV ₁	8.3 ± 8.9	7.1 ± 8.2	6.3 ± 7.5	6.3 ± 7.6	6.0 ± 7.7	<0.0001
Adjusted relative rate of decline, ml/yr (mean ± SE)	11.02 ± 3.30	3.48 ± 3.21	0.14 ± 3.16	3.15 ± 3.14	1 (Reference)	0.0033*
Decline in FEV ₁ from Year 5 to Year 11						
ml/yr	58 ± 41	54 ± 45	53 ± 44	51 ± 44	50 ± 47	0.0012
% of baseline FEV ₁ (Year 5)	38.7 ± 38.7	33.7 ± 37.9	32.1 ± 37.5	31.4 ± 37.5	29.5 ± 36.9	<0.0001
Adjusted relative rate of decline, ml/yr (mean ± SE)	8.03 ± 2.44	3.89 ± 2.30	3.69 ± 2.26	1.16 ± 2.25	1 (Reference)	0.0006*

A positive number denotes faster decline in FEV₁. Values are presented as mean ± SD, except for adjusted relative rate of decline.

* Adjusted for age, sex, race, smoking status, body mass index, airway reactivity, and baseline FEV₁

[†] P value based on mixed linear models taking into account all spirometry data from Year 2 to Year 11, adjusted for age, sex, race, smoking status, body mass index, airway reactivity, and baseline FEV₁

obesity, resolving the conflicting data that had been previously reported (11, 19). In our study, participants with higher serum levels of CC-16 were less likely to have increased airway reactivity determined on a methacholine provocation test. Adjustments for these and other possible confounders made little impact on the overall findings with respect to the association of the CC-16 with the rate of decline in FEV₁. It is important to note that, although annual rate of decline in FEV₁ is an indicator of disease progression in COPD, it is highly variable within and across patients with COPD (5–7). Thus, large sample sizes are required to study effects of intervention on rate of decline in FEV₁.

CC-16 is a 16-kD protein that is primarily secreted from the lungs (20). Because it is a small and readily diffusible protein, it leaks from the respiratory tract into systemic circulation across the air–blood barrier. Once CC-16 gets into serum, it is rapidly cleared by glomerular filtration (18). Thus, serum CC-16 levels are increased in patients with poor renal function or in those with increased lung permeability (18, 21). Serum levels of CC-16 are stable and reproducible over time (11) and, in healthy individuals, serum CC-16 levels are significantly related to CC-16 levels in bronchoalveolar lavage fluid (22). Interestingly, serum and bronchoalveolar lavage concentrations of CC-16 are decreased in persistent smokers, which is related to reduced numbers of intact club cells in the lungs (17, 23).

Although previous *in vivo* findings have shown that CC-16 plays a protective role against pulmonary irritants and, in general, has anti-inflammatory properties (18), CC-16^{−/−} mice did not have increased susceptibility to cigarette-induced emphysema or small airway remodeling over 6 months of cigarette exposure in our experiments. Prior short-term studies have shown that CC-16 deficiency enhanced susceptibility to oxidative stress response after induction of proinflammatory cytokine gene expression (24, 25), and CC-16^{−/−} mice had an augmented inflammatory response in the lung with acute viral infection or LPS exposure (26, 27). To our knowledge, our study is the first to investigate a biological role of CC-16 in a smoking-induced COPD model, and, despite these short-term data, we failed to demonstrate any significant augmentation of emphysema or small airway remodeling with chronic cigarette exposure in CC-16^{−/−} mice. It should be noted that our animal study was relatively small and, as such, small differences in pathologic processes related to cigarette smoking between CC-16^{−/−} and wild-type mice could have been missed. Thus, we cannot fully discount a modest role of CC-16 in the pathogenesis of COPD or its possible role in more advanced stages of COPD, which cannot be adequately modeled in mice (28). Additional investigations with larger sample sizes are

needed to further clarify the possible pathogenic role of CC-16 in COPD.

There were other limitations to this study. First, the LHS cohort recruited only patients who had mild or moderate disease. Thus, our findings may not be applicable to severe stages. Notwithstanding, because the rate of FEV₁ decline is generally faster in patients with mild or moderate disease compared with those with more advanced disease (6), results of the present study are likely to be highly relevant for future interventional studies designed to modify rate of decline in lung function. Second, LHS did not use thoracic computed tomography to phenotype COPD. Thus, the relationship between serum CC-16 and extent of emphysema or other morphological phenotypes of COPD is unknown. Previously, the ECLIPSE investigators failed to find a significant association between severity of emphysema on computed tomography scans and serum CC-16 levels in individuals with COPD (11). Third, as we did not have serial measurements of CC-16, we could not determine intraindividual variation of serum CC-16 levels over time. Fourth, although serum CC-16 levels were significantly associated

TABLE 3. AREA UNDER THE CURVE VALUES FROM RECEIVER OPERATOR CHARACTERISTIC GENERATED FOR VARIABLES WITH CLUB (CLARA) CELL PROTEIN-16 TO PREDICT RAPID DECLINE OF FEV₁

Variables	AUC (95% CI)
Smoking status	0.6270 (0.6088–0.6453)
Smoking status + airway reactivity	0.6498 (0.6306–0.6690)*
Smoking status + airway reactivity + baseline FEV ₁ (Year 2)	0.6588 (0.6399–0.6778)
Smoking status + airway reactivity + baseline FEV ₁ (Year 2) + sex	0.6597 (0.6407–0.6786)
Smoking status + airway reactivity + baseline FEV ₁ (Year 2) + sex + age	0.6641 (0.6452–0.6830)
Smoking status + airway reactivity + baseline FEV ₁ (Year 2) + sex + age + BMI	0.6641 (0.6452–0.6830)
Smoking status + airway reactivity + baseline FEV ₁ (Year 2) + sex + age + BMI + Race	0.6645 (0.6456–0.6834)
Smoking status + airway reactivity + baseline FEV ₁ (Year 2) + sex + age + BMI + Race + log CC-16	0.6695 (0.6507–0.6884) [†]

Definition of abbreviations: AUC = area under the curve; BMI = body mass index; CC-16 = Club (Clara) cell protein-16; CI = confidence interval.

Rapid decline of FEV₁ is defined here as ≥40 ml/yr.

* P < 0.0001, P value for comparison with model of previous step.

[†] P = 0.0335, P value for comparison with model of previous step.

Figure 3. Morphometric measures. (A) Measures of mean airspace size (mean linear intercept) as a measure of emphysema. (B and C) Measures of bronchiolar wall thickness and bronchiolar wall area/unit basement membrane as indicators of small airway remodeling. Bars represent means and the error bars represent SEM ($n = 5-7$ animals per group). $*P < 0.05$. NS = nonsignificant.

with smoking status, there was large overlap between serum CC-16 levels in the continued smokers and sustained quitters. It is also uncertain whether serum CC-16 concentrations are modifiable by

therapeutic intervention. Finally, it should be noted that, although the relationship between CC-16 and rate of decline in FEV₁ was statistically significant, the overall strength of the association was

Figure 4. Tissue inflammatory cells. (A) Tissue macrophages. (B) Tissue neutrophils. Bars represent means and the error bars represent SEM ($n = 5-7$ animals per group). $*P < 0.05$. NS = nonsignificant.

relatively weak. Thus, it is unlikely that CC-16 can be used as a standalone biomarker to select out rapid progressors.

In summary, the findings of the present study suggest that serum CC-16 is significantly related to decline in lung function. These data raise the possibility of using this biomarker in concert with clinical risk factors (such as cigarette smoking and baseline FEV₁) to select and enrich future COPD cohorts for “rapid” progressors, and thus enhance the statistical power and precision of future interventional studies of COPD. Additional human and animal studies are required to validate these findings and to determine the precise role of CC-16 as a biomarker of disease progression in COPD.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the Lung Health Study Investigators and the participants of the Lung Health Study.

References

- Fletcher C, Peto R. The natural history of chronic airflow obstruction. *BMJ* 1977;1:1645–1648.
- Global Initiative for Chronic Obstructive Lung Disease. Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease [updated 2013 Feb 1; accessed 2013 April 1]. Available from: <http://www.goldcopd.org>
- Lopez AD, Shibuya K, Rao C, Mathers CD, Hansell AL, Held LS, Schmid V, Buist S. Chronic obstructive pulmonary disease: current burden and future projections. *Eur Respir J* 2006;27:397–412.
- Agusti A, Calverley PM, Celli B, Coxson HO, Edwards LD, Lomas DA, MacNee W, Miller BE, Rennard S, Silverman EK, *et al.*; Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) investigators. Characterisation of COPD heterogeneity in the ECLIPSE cohort. *Respir Res* 2010;11:122.
- Vestbo J, Edwards LD, Scanlon PD, Yates JC, Agusti A, Bakke P, Calverley PM, Celli B, Coxson HO, Crim C, *et al.*; ECLIPSE Investigators. Changes in forced expiratory volume in 1 second over time in COPD. *N Engl J Med* 2011;365:1184–1192.
- Casanova C, de Torres JP, Aguirre-Jaime A, Pinto-Plata V, Marin JM, Cordoba E, Baz R, Cote C, Celli BR. The progression of chronic obstructive pulmonary disease is heterogeneous: the experience of the BODE cohort. *Am J Respir Crit Care Med* 2011;184:1015–1021.
- Nishimura M, Makita H, Nagai K, Konno S, Nasuhara Y, Hasegawa M, Shimizu K, Betsuyaku T, Ito YM, Fuke S, *et al.*; Hokkaido COPD Cohort Study Investigators. Annual change in pulmonary function and clinical phenotype in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2012;185:44–52.
- Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* 2006;3:e442.
- Lakind JS, Holgate ST, Ownby DR, Mansur AH, Helms PJ, Pyatt D, Hays SM. A critical review of the use of Clara cell secretory protein (CC16) as a biomarker of acute or chronic pulmonary effects. *Biomarkers* 2007;12:445–467.
- Broeckaert F, Bernard A. Clara cell secretory protein (CC16): characteristics and perspectives as lung peripheral biomarker. *Clin Exp Allergy* 2000;30:469–475.
- Lomas DA, Silverman EK, Edwards LD, Miller BE, Coxson HO, Tal-Singer R; Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) Investigators. Evaluation of serum CC-16 as a biomarker for COPD in the ECLIPSE cohort. *Thorax* 2008;63:1058–1063.
- Man SF, Xing L, Connett JE, Anthonisen NR, Wise RA, Tashkin DP, Zhang X, Vessey R, Walker TG, Celli BR, *et al.* Circulating fibronectin to C-reactive protein ratio and mortality: a biomarker in COPD? *Eur Respir J* 2008;32:1451–1457.
- Wise RA, Kanner RE, Lindgren P, Connett JE, Altose MD, Enright PL, Tashkin DP; Lung Health Study Research Group. The effect of smoking intervention and an inhaled bronchodilator on airways reactivity in COPD: the Lung Health Study. *Chest* 2003;124:449–458.
- Sin DD, Miller BE, Duvoix A, Man SF, Zhang X, Silverman EK, Connett JE, Anthonisen NA, Wise RA, Tashkin D, *et al.*; ECLIPSE Investigators. Serum PARC/CCL-18 concentrations and health outcomes in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2011;183:1187–1192.
- Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, Conway WA Jr, Enright PL, Kanner RE, O'Hara P, *et al.* Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV₁. The Lung Health Study. *JAMA* 1994;272:1497–1505.
- Anthonisen NR, Connett JE, Murray RP. Smoking and lung function of Lung Health Study participants after 11 years. *Am J Respir Crit Care Med* 2002;166:675–679.
- Shijubo N, Itoh Y, Yamaguchi T, Shibuya Y, Morita Y, Hirasawa M, Okutani R, Kawai T, Abe S. Serum and BAL Clara cell 10 kDa protein (CC10) levels and CC10-positive bronchiolar cells are decreased in smokers. *Eur Respir J* 1997;10:1108–1114.
- Broeckaert F, Clippe A, Knoop B, Hermans C, Bernard A. Clara cell secretory protein (CC16): features as a peripheral lung biomarker. *Ann N Y Acad Sci* 2000;923:68–77.
- Nomori H, Horio H, Takagi M, Kobayashi R, Hirabayashi Y. Clara cell protein correlation with hyperlipidemia. *Chest* 1996;110:680–684.
- Hermans C, Bernard A. Lung epithelium-specific proteins: characteristics and potential applications as markers. *Am J Respir Crit Care Med* 1999;159:646–678.
- Doyle IR, Hermans C, Bernard A, Nicholas TE, Bersten AD. Clearance of Clara cell secretory protein 16 (CC16) and surfactant proteins A and B from blood in acute respiratory failure. *Am J Respir Crit Care Med* 1998;158:1528–1535.
- Bernard A, Marchandise FX, Depelchin S, Lauwerys R, Sibille Y. Clara cell protein in serum and bronchoalveolar lavage. *Eur Respir J* 1992;5:1231–1238.
- Bernard AM, Roels HA, Buchet JP, Lauwerys RR. Serum Clara cell protein: an indicator of bronchial cell dysfunction caused by tobacco smoking. *Environ Res* 1994;66:96–104.
- Mango GW, Johnston CJ, Reynolds SD, Finkelstein JN, Plopper CG, Stripp BR. Clara cell secretory protein deficiency increases oxidant stress response in conducting airways. *Am J Physiol* 1998;275:L348–L356.
- Johnston CJ, Mango GW, Finkelstein JN, Stripp BR. Altered pulmonary response to hyperoxia in Clara cell secretory protein deficient mice. *Am J Respir Cell Mol Biol* 1997;17:147–155.
- Harrod KS, Mounday AD, Stripp BR, Whitsett JA. Clara cell secretory protein decreases lung inflammation after acute virus infection. *Am J Physiol* 1998;275:L924–L930.
- Snyder JC, Reynolds SD, Hollingsworth JW, Li Z, Kaminski N, Stripp BR. Clara cells attenuate the inflammatory response through regulation of macrophage behavior. *Am J Respir Cell Mol Biol* 2010;42:161–171.
- Churg A, Sin DD, Wright JL. Everything prevents emphysema: are animal models of cigarette smoke-induced chronic obstructive pulmonary disease any use? *Am J Respir Cell Mol Biol* 2011;45:1111–1115.