Sputum Biomarkers of Inflammation and Lung Function Decline in Children with Cystic Fibrosis

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Rationale: Progressive lung function decline is a defining feature of cystic fibrosis (CF). Because airway inflammation plays a central role in CF lung disease, inflammatory biomarkers that can be used to monitor disease activity would be valuable.

Objectives: Examine longitudinal relationships between sputum biomarkers and lung function.

Methods: In this prospective, longitudinal cohort study, sputum induction was performed annually over 3 years in 35 children with CF. Sputum was assayed for mediators related to proteolysis and a panel of inflammatory cytokines.

Measurements and Main Results: Sputum neutrophil elastase, tissue inhibitor of metalloproteinase-1, and TNF- α increased over time, whereas neutrophil elastase antiprotease complexes (NEAPCs) and secretory leukoprotease inhibitor (SLPI) significantly decreased over time. Higher detectable baseline neutrophil elastase was associated with more rapid lung function decline. Similar results for neutrophil elastase were observed in a validation cohort. When categorizing subjects as "rapid" or "slow" decliners, logistic regression demonstrated that the initial measurement of neutrophil elastase had the highest individual predictive value for subsequent lung function decline, whereas neutrophil elastase, IL-8, and IL-6 had the highest combined predictive value. Lung function decline was associated with increases in neutrophil counts, neutrophil elastase, and IL-1 β and declines in NEAPCs and SLPI.

Conclusions: In children with CF, a single determination of sputum biomarkers, particularly neutrophil elastase, has predictive value for subsequent lung function decline, and longitudinal changes in sputum inflammatory biomarkers are related to lung function changes. Based on our results, sputum neutrophil elastase was the most informative biomarker to monitor disease activity.

Keywords: cystic fibrosis; pulmonary function; sputum; inflammation; neutrophil elastase

Progressive lung function decline is a defining feature of cystic fibrosis (CF) pulmonary disease (1). Yet, the rate of lung function

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Data from cross-sectional studies support associations between sputum markers of inflammation and clinical outcomes, including lung function, in cystic fibrosis. However, longitudinal analyses are essential for the validation of biomarkers of inflammation as correlates of disease severity.

What this Study Adds to the Field

A single determination of sputum inflammatory biomarkers, particularly neutrophil elastase, has predictive value for subsequent lung function decline in cystic fibrosis, and longitudinal changes in sputum biomarkers are related to lung function changes.

decline in CF varies considerably even among individuals carrying the same genetic mutations (2). It is unclear which children are at risk for earlier and more severe lung disease and who will experience a more rapid lung function decline. Because airway inflammation plays a central role in CF lung disease, biomarkers of inflammation that can be used to monitor disease activity would be extremely valuable to recognize patients most in need of urgent intervention. Inflammatory biomarkers may also provide further insight into the pathophysiology of CF lung disease, identify treatment targets, and rapidly assess response to current or potential new therapies.

There is a paucity of data regarding airway inflammation in school-age children with mild lung disease (3, 4). Proteolytic activity is believed to be responsible for causing most of the bronchiectasis in CF (5, 6). There is limited yet compelling evidence from small, single-center studies supporting an association between proteases and lung function measurements (7, 8), chest radiograph scores (9–11), and illness severity scores (e.g., Shwachman-Kulczycki score) (9, 10) in children and adults with CF. Furthermore, there are data that show the correlations between sputum protease levels and lung function are statistically significant across a larger diverse CF population (12). Although cross-sectional studies are most commonly reported in the literature, longitudinal analyses are essential for the validation of biomarkers of inflammation as correlates of disease severity and progression. Because the greatest rate of decline in lung function occurs in late childhood and adolescent years (13), it stands to reason that changes in airway inflammation may play a significant role in this decline.

In this prospective longitudinal study, we performed sputum inductions annually over 3 years during times of clinical stability in a cohort of school-age children with CF to determine the predictive ability of a single measurement of sputum biomarkers for more rapid lung function decline and to investigate relationships

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between changes in markers of airway inflammation and lung function. We hypothesized that children with CF with more pronounced airway proteolytic activity will have a greater degree of functional lung impairment and changes in proteolytic markers over time will relate to changes in lung function. The goal of this study was to identify proteolytic biomarkers that are associated with lung disease progression and predictive of future clinical course in children with CF. Some of the results of these studies have been reported in the form of an abstract (14).

METHODS

Study Cohorts and Design

In this prospective longitudinal cohort study, we recruited 35 children with CF between the ages of 6 and 15 years at the time of study entry from among our outpatient CF clinics. These children were studied annually over 3 years (four study visits) during times of clinical stability, defined by clinical impression and having had no recent hospitalizations or significant changes in antibiotic regimen within 2 weeks of each clinic visit. At these four visits, spirometry was performed according to American Thoracic Society guidelines (15). Functional indices included FEV₁, FVC, and mid-volume forced expiratory flows (FEF_{25–75}). All spirometric values were expressed as percent of predicted normal using reference equations (16). After spirometry, sputum induction was performed according to a standard operating procedure as previously described (4). The number of courses of intravenous antibiotics in the year preceding each of the study visits, used as a surrogate of pulmonary exacerbations, was captured. A validation study was also performed in 10 independent clinically stable children with CF who had undergone a single sputum induction for microbiome investigations. Spirometric data were collected on this validation cohort at the time of the sputum induction and during a follow-up period of 2.5 to 3 years. This study was conducted with Institutional Review Board approval between 2004 and 2009. Written informed consent was obtained for all enrolled subjects.

Sputum Processing and Analysis

During the sputum induction procedure, sputum was collected into two containers. One specimen was submitted for comprehensive microbiology per CF consensus guidelines (17). The second specimen was processed within 20 minutes of collection for cytology (including total cell counts and neutrophils) and measurement of inflammatory markers using a standard operating procedure (4) in the Pediatric Clinical Translational Research Center Core Laboratory at Children's Hospital Colorado. Only samples that weighed greater than 0.5 g and had less than 80% squamous cells by cytologic examination were considered adequate and included for further analysis. After homogenization of the sputum using sterile 0.1% dithiothreitol (10% Sputolysin; Calbiochem-Novabiochem Corp., San Diego, CA), the liquefied sputum was vigorously centrifuged twice. One portion of the supernatant was treated with protease inhibitors, phenylmethylsulfonylfluoride, and ethylenediamine tetraacetic acid (Sigma Diagnostics, St. Louis, MO) to minimize proteolytic activity. The remaining portion was left untreated. Supernatants were frozen at -70°C for later analysis of inflammatory markers.

The proteases (neutrophil elastase, matrix metalloproteinase [MMP]-2, and MMP-9) and antiproteases (neutrophil elastase $\alpha 1$ antiprotease complexes [NEAPCs], secretory leukoprotease inhibitor [SLPI], and tissue inhibitor of metalloproteinase-1 [TIMP-1]) were measured in thawed, untreated supernatants, whereas the cytokines IL-1 β , IL-6, IL-8, IL-17, and TNF- α were quantified in supernatants treated with protease inhibitors. Free neutrophil elastase activity was quantified by a spectrophotometric assay based on the hydrolysis of the specific substrate MeO-suc-Ala-Ala-Pro-Ala-*p*-nitroanilide (Sigma Chemical Co, St. Louis, MO), whereas MMP-9 activity, MMP-2, SLPI, and TIMP-1 were measured by commercially available ELISA kits (Quantikine; R&D Systems, Minneapolis, MN). NEAPC was also determined through a commercially available ELISA kit (Alpco Diagnostics, Windham, NH). The cytokine panel was quantified on a Luminex multiplex platform using commercially available reagents (R&D Systems). The lower limits of detection (LOD) for these assays were: neutrophil elastase,

0.5 $\mu\text{g/ml}$; MMP-2, 0.8 ng/ml; MMP-9, 0.3 ng/ml; TIMP-1, 15.6 ng/ml; NEAPC, 16.0 ng/ml; SLPI, 0.125 ng/ml, IL-1 β , 2.5 pg/ml; IL-6, 2.0 pg/ml; IL-8, 1.0 pg/ml; IL-17, 2.0 pg/ml; TNF- α , 2.0 pg/ml.

Statistical Analysis

Descriptive statistics were used to characterize the demographic and baseline characteristics and included the mean and standard deviation and median and interquartile range where specified. To characterize the rate of change in FEV₁% predicted during the study period, individual slope estimates for FEV₁% predicted were calculated using random coefficient models with separate population average coefficients for male and female subjects (18). A simple multivariate regression model was fit to the subject specific slopes obtained using the random coefficients approach to identify independent baseline clinical risk factors associated with changes in FEV₁% predicted. Clinical risk factors included in the model were baseline age, gender, CF genotype, FEV₁% predicted, growth indices (including body mass index), and respiratory tract culture results for *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

All sputum biomarker variables were log₁₀ transformed and centered at 1, with some values being left-censored if they were below the LOD of the respective assays. To investigate changes in sputum biomarkers over time, mixed models, which accounted for left-censoring, were used to estimate the least square means, corresponding 95% confidence intervals, and linear trend (i.e., slope) of individual biomarkers (19). SAS proc NLMIXED was used with the Quasi-Newton optimization algorithm to fit these models (20). The time effect was entered as a categorical variable in the models, and the linear trend over time was calculated and tested.

The relationships between FEV₁% predicted and each of the sputum biomarkers were estimated using a repeated measures model in which FEV₁% predicted from all four visits was the primary outcome, and each biomarker was included as a predictor variable. An indicator variable was used to address the left-censoring of the biomarker values (12). In a two-stage approach, a simple multivariate regression model was fit to the subject specific slopes obtained from the random coefficients model to identify independent baseline clinical risk factors (including sputum biomarker values) associated with changes in FEV₁% predicted. The FEV₁% predicted slope values were categorized using a cutoff of -2% predicted per year to classify patients as "rapid decliners," defined as a rate of decline in FEV₁ greater than 2% per year, or as "slow decliners," declining at a rate less than 2% per year. This dichotomous variable was used as the outcome in a logistic regression to obtain estimates of the predictive ability of the baseline sputum biomarker values, using receiver operating characteristic curves.

For the validation cohort, a random coefficients model was used to characterize the rate of change in FEV₁% predicted during the follow-up period. Because neutrophil elastase was the most informative biomarker in the main study cohort, this was the only inflammatory marker measured in the sputum samples from the validation cohort. Validation consisted of two steps: (1) determining the correlation between baseline neutrophil elastase (log-transformed) and annual change in FEV₁% predicted and (2) examining the predictive ability of the baseline sputum neutrophil elastase values for being a "rapid decliner."

Spearman's nonparametric rank correlation coefficients were calculated to assess the correlations between changes in sputum biomarkers and changes in FEV₁% predicted. *P* values less than 0.05 were considered statistically significant for all analyses. All analyses were performed with SAS Version 9.2 (SAS, Cary, NC).

RESULTS

Clinical Characteristics of Study Subjects

The baseline clinical characteristics of our study subjects are summarized in Table 1. At the time of study enrollment, subjects who comprised the main study cohort ranged in age from 6 to 15 years. The distribution of genotypes in our study cohort closely reflected the genotype distribution in our clinic population. Although the FEV₁% predicted ranged from 62 to 124%, most of our subjects had normal or mildly abnormal

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY COHORTS AT TIME OF BASELINE STUDY VISIT

Characteristics	Main Study Cohort (n = 35)	Validation Cohort (n = 10)
Gender (M:F)	20:15	5:5
Age, yr	11.1 (2.6)	12.6 (4.0)
CF genotype		
ΔF508/ΔF508	14 (40%)	5 (50%)
ΔF508/other	15 (43%)	5 (50%)
Other/other	6 (17%)	—
FVC% predicted	95.5 (17.6)	114.8 (1.2)
FEV ₁ % predicted	95.3 (15.0)	93.8 (23.8)
Weight, percentile	37.7 (24.9)	36.9 (27.7)
Height, percentile	34.9 (25.7)	28.4 (21.9)
Body mass index, percentile	42.3 (25.0)	49.3 (27.4)
CF pathogens, n (%) [*]		
<i>Pseudomonas aeruginosa</i>	6 (17%)	5 (50%)
<i>Staphylococcus aureus</i>	16 (46%)	5 (50%)
<i>Stenotrophomonas maltophilia</i>	6 (17%)	2 (20%)
<i>Aspergillus fumigatus</i> (or other molds)	9 (26%)	4 (40%)
<i>Mycobacterium avium</i> complex or <i>Mycobacterium abscessus</i>	2 (6%)	—

Definition of abbreviation: CF = cystic fibrosis.

Results are number (percent) or mean (SD) unless otherwise noted.

^{*}Respiratory culture data are available from 32 of 35 (91%) main study cohort subjects who expectorated induced sputum samples for microbial analysis at their baseline visit.

lung function. The growth indices of our study cohort closely reflected those of our entire clinic population. Thirty-two subjects (91%) expectorated adequate induced sputum samples for microbial analysis at their baseline visit. CF-specific pathogens were detected in 26 of 32 specimens (Table 1). The validation cohort was slightly older than the main study cohort but had similar lung function indices.

Lung Function Change over Time in the Main Study Cohort

The mean change in FEV₁% predicted for the entire cohort was −2.1% predicted per year, ranging from an improvement of 0.6% predicted per year to a decline of −5.4% predicted per year. The mean changes in FVC and FEF_{25–75}% predicted for the entire cohort were 0.1% and −5.8% predicted per year, respectively. For the remainder of this report, we focus on changes in FEV₁% predicted per year because it was the main outcome variable in this study. In our study cohort, male subjects experienced a greater annual decline in FEV₁% predicted compared with female subjects (mean [SE]: male subjects, −2.80 [0.21]; female subjects, −1.27 [0.29]; *P* < 0.01). In comparing baseline risk factors between genders, the only difference observed was a higher baseline FEV₁% predicted in the male subjects compared with the female subjects (mean [95% CI]: male subjects: 100.4 [95.2–105.6]; female subjects: 88.5 [79.0–98.0]) (see Table E1 in the online supplement).

In a multivariate regression model examining which independent baseline clinical risk factors were associated with changes in FEV₁% predicted, decline in FEV₁ was found to be associated with gender, and baseline FEV₁% predicted and marginally related to age at baseline study visit (see Table 2). More specifically, higher baseline FEV₁, male gender, and advancing age were the factors associated with more rapid rates of FEV₁ decline among our study cohort. There was no significant interaction between male gender and baseline FEV₁% predicted (estimate [SE]: 0.01 [0.03]; *P* = 0.64). Infection with *P. aeruginosa* and *S. aureus* were not associated with changes in FEV₁% predicted.

Longitudinal Changes in Sputum Biomarkers of Airway Inflammation

Of the 132 sputum inductions performed in our study cohort over four study visits, 120 (91%) yielded adequate induced sputum

samples (defined as weighing > 0.5 g and containing at least 20% nonsquamous cells and at least 20% neutrophils) for further analysis. A few of these expectorated sputum samples were not of sufficient volume to perform all of the indicated biochemical measurements and quantitative bacterial cultures. For two of the biomarkers, IL-17 and MMP-2, the majority of sputum samples had undetectable values or values below the limits of detection of these assays. All missing values and all IL-17 and MMP-2 data were excluded from further analysis.

Descriptive statistics for the logarithmically transformed concentrations of each sputum biomarker across the four study visits are listed in Table E2. Of all the biomarkers, only IL-1β and IL-8 were detected in amounts greater than the limits of detection (LOD) of the respective assays in all adequate sputum samples tested. All of the other biomarkers had at least some proportion of their values below the LOD of the assay (Table E2). Of the two proteases, neutrophil elastase was present in higher concentrations than MMP-9. For the antiproteases, SLPI was present in the highest amounts. Regarding the cytokines, IL-8 was detected in the largest amounts, whereas TNF-α was present in the lowest quantities.

Changes in sputum biomarkers of airway inflammation over time are shown in Table 3 and were estimated after accounting

TABLE 2. INDEPENDENT EFFECTS OF BASELINE CLINICAL RISK FACTORS ON CHANGE IN FEV₁% PREDICTED

Baseline Risk Factor	Full Model (<i>R</i> ² = 0.59)		Reduced Model (<i>R</i> ² = 0.56)	
	ParamEst (SE)	<i>P</i> Value	ParamEst (SE)	<i>P</i> Value
Age	−0.14 (0.07)	0.06	−0.16 (0.06)	0.01
Gender (female)	1.9 (0.4)	<0.01	1.8 (0.3)	<0.01
FEV ₁ % predicted	0.03 (0.01)	0.04	0.03 (0.01)	0.01
CF genotype (high risk) [*]	−0.01 (0.39)	0.97		
BMI percentile	0.01 (0.01)	0.34		
<i>Pseudomonas aeruginosa</i> infection [†]	−0.26 (0.42)	0.55		
<i>Staphylococcus aureus</i> infection [†]	−0.28 (0.42)	0.51		

^{*}Subjects were classified as having a “high risk” genotype if they had two identified class I, II or III CF mutations. Subjects with at least one class IV, V, or unidentified mutation were classified as having a “low risk” genotype.

[†]Infection status was based on having a positive respiratory culture for *P. aeruginosa* or *S. aureus* during any of the four study visits.

TABLE 3. INDUCED SPUTUM LEVELS OF INFLAMMATORY BIOMARKERS AND LINEAR TREND OVER FOUR ANNUAL STUDY VISITS*

Biomarker	Visit 1	Visit 2	Visit 3	Visit 4	Linear Trend
Total (log) neutrophils	7.6 (7.4 to 7.9)	7.6 (7.4 to 7.8)	7.5 (7.3, 7.8)	7.4 (7.2 to 7.7)	−0.3 (−0.7 to <0.1)
% Neutrophils	63 (57 to 70)	60 (53 to 67)	55 (48 to 62)	64 (57 to 71)	−0.02 (−0.15 to 0.11)
Elastase log, µg/ml	1.2 (1.0 to 1.4)	1.4 (1.2 to 1.6)	1.3 (1.1 to 1.5)	1.6 (1.4 to 1.8)	0.5 (0.2 to 0.8)
MMP-9 log, ng/ml	3.5 (3.3 to 3.7)	3.6 (3.4 to 3.8)	3.8 (3.6 to 3.9)	3.5 (3.3 to 3.7)	0.1 (−0.3 to 0.5)
NEAPC log, ng/ml	2.4 (2.2 to 2.5)	2.2 (2.0 to 2.3)	2.2 (2.0 to 2.3)	2.1 (1.9 to 2.2)	−0.4 (−0.7 to −0.1)
SLPI log, ng/ml	3.9 (3.8 to 4.1)	3.7 (3.6 to 3.8)	3.7 (3.6 to 3.8)	3.7 (3.6 to 3.8)	−0.3 (−0.5 to −0.1)
TIMP-1 log, ng/ml	2.5 (2.4 to 2.6)	2.6 (2.4 to 2.7)	2.7 (2.6 to 2.8)	2.7 (2.6 to 2.9)	0.5 (0.3 to 0.7)
IL-1β log, pg/ml	3.0 (2.8 to 3.2)	2.9 (2.6 to 3.1)	2.9 (2.6 to 3.1)	3.0 (2.8 to 3.3)	0.1 (−0.3 to 0.4)
IL-6 log, pg/ml	1.7 (1.4 to 2.0)	1.5 (1.2 to 1.8)	1.7 (1.4 to 2.0)	1.8 (1.4 to 2.1)	0.2 (−0.3 to 0.7)
IL-8 log, pg/ml	4.7 (4.6 to 4.8)	4.8 (4.6 to 4.9)	4.8 (4.6 to 4.9)	4.7 (4.6 to 4.9)	0.1 (−0.2 to 0.3)
TNF-α log, pg/ml	0.9 (0.7 to 1.1)	1.3 (1.1 to 1.5)	1.4 (1.2 to 1.6)	1.7 (1.5 to 1.9)	1.2 (0.7 to 1.6)

Definition of abbreviations: NEAPC = neutrophil elastase antiprotease complex; SLPI = secretory leukoprotease inhibitor; TIMP-1 = tissue inhibitor of metalloproteinase.

* Data presented as least square means with 95% confidence intervals. The linear trend (i.e., slope) is based on a standard mixed model, excluding the influence of censoring. Bold values indicate a statistically significant change over time (95% CI does not include zero).

for left-censoring due to values below the LOD. Significant changes were observed in neutrophil elastase, NEAPC, SLPI, TIMP-1, and TNF-α. Specifically, neutrophil elastase, TIMP-1, and TNF-α increased over time, whereas NEAPC and SLPI showed a significant linear decrease over the study period (Table 3; see Figure E1 in online supplement). Total neutrophil counts did not change significantly over time. The protease MMP-9 did not significantly change; nor did any of the other cytokines (except TNF-α). Examining relationships between changes in sputum biomarkers, change in neutrophil elastase correlated positively with changes in total neutrophil counts ($r = 0.54$), percent neutrophils ($r = 0.46$), IL-8 ($r = 0.73$), IL-1β ($r = 0.63$), and MMP-9 ($r = 0.50$) and correlated negatively with NEAPC ($r = -0.53$) and SLPI ($r = -0.58$).

Relationships between Baseline Determination of Sputum Biomarkers and Change in Lung Function

The associations between baseline sputum biomarker measurements and subsequent annual rate of change in FEV₁% predicted are shown in Table 4. The only significant relationship observed was between baseline neutrophil elastase and annual rate of change in FEV₁% predicted. Higher detectable baseline neutrophil elastase concentrations were associated with greater change in FEV₁% predicted (Figure 1). In other words, subjects with detectable and higher neutrophil elastase levels at the initial study visit tended to experience a more rapid decline in FEV₁. A one unit increase in (log) neutrophil elastase corresponded

to a 1.1% predicted per year decline in FEV₁. When all sputum biomarker values were included (those above and below the LOD of the assays), the associations between baseline neutrophil elastase measurements and subsequent annual rate of change in FEV₁% predicted remained statistically significant with a similar slope estimate (estimate [SE] = -1.0 [0.5]; $P = 0.04$). The effects of baseline clinical risk factors on baseline sputum neutrophil elastase levels can be found in the online supplement.

Baseline sputum biomarker measurements were included in the multivariate regression model (Table 2) to determine whether sputum biomarkers add value to baseline clinical risk factors for predicting changes in FEV₁% predicted over time. Measurements of sputum neutrophil elastase and NEAPC offered slight added value for determining subsequent changes in FEV₁% predicted (Table E3). Infection with *P. aeruginosa* and *S. aureus* was not predictive of subsequent changes in FEV₁% predicted.

To further examine the predictive ability of sputum biomarkers, we categorized our subjects as “rapid decliners” ($n = 20$) or “slow decliners” ($n = 15$). A logistic regression model revealed that the initial measurement of neutrophil elastase had the highest individual predictive value for subsequent lung function decline ($c = 0.64$); neutrophil elastase and IL-8 had the highest combined predictive value for any subset of two biomarkers ($c = 0.75$); and neutrophil elastase, IL-8, and IL-6 had the highest combined predictive value for any subset of three markers ($c = 0.82$) (Figure 2).

TABLE 4. CORRELATIONS BETWEEN FEV₁% PREDICTED SLOPE ESTIMATES AND BASELINE MEASUREMENTS OF SPUTUM BIOMARKERS OF INFLAMMATION*

Biomarker	Intercept Est (SE)	Censored Est (SE)	Linear Trend Est (SE)	P Value
Elastase log, µg/ml	−0.78 (0.7)	−1.12 (0.9)	−1.12 (0.5)	0.04
MMP-9 log, ng/ml	−2.59 (2.1)	−0.28 (2.5)	0.12 (0.6)	0.85
NEAPC log, ng/ml	−6.42 (3.0)	4.21 (3.0)	1.75 (1.2)	0.16
SLPI log, ng/ml	−5.01 (3.0)	—	0.72 (0.8)	0.36
TIMP-1 log, ng/ml	−2.07 (2.2)	0.62 (2.4)	−0.07 (0.9)	0.94
IL-1β log, pg/ml	−0.99 (1.1)	—	−0.40 (0.4)	0.28
IL-6 log, pg/ml	−2.12 (1.4)	0.14 (1.5)	−0.07 (0.7)	0.92
IL-8 log, pg/ml	−2.07 (2.9)	—	−0.03 (0.6)	0.97
TNF-α log, pg/ml	−1.81 (0.7)	−0.38 (0.8)	—	—

Definition of abbreviations: MMP-9 = matrix metalloproteinase 9; NEAPC = neutrophil elastase antiprotease complex; SLPI = secretory leukoprotease inhibitor; TIMP-1 = tissue inhibitor of metalloproteinase.

* The linear trend indicates whether the relationship is positive or negative and indicates the magnitude of the relationship. The P value corresponds to the test of whether the slope is significantly different from 0, adjusting for all censored values (i.e., those below the limit of detection of the respective assays).

The bold value indicates a statistically significant correlation.

Figure 1. Higher baseline neutrophil elastase concentrations were associated with faster rates of decline in FEV₁% predicted. For the purposes of graphical display, nondetectable values for neutrophil elastase were plotted at the limit of detection (LOD), but these values could lie in any interval between zero and the LOD. The plot displays the regression line corresponding to the detectable values.

Relationships between Serial Measurements of Sputum Biomarkers and Change in Lung Function

The correlations between changes in sputum biomarker measurements and annual rate of change in FEV₁% predicted are shown in Table 5 (Figure 3). Changes in FEV₁% predicted correlated significantly with changes in sputum total neutrophil counts, neutrophil elastase, and IL-1 β , and near-significant associations were observed with NEAPC, SLPI, and IL-8. More specifically, a decline in FEV₁% predicted was significantly associated with increases in neutrophil counts, neutrophil elastase, and IL-1 β and was marginally associated with declines in NEAPC and SLPI and rising IL-8. The strongest correlation was between changes in FEV₁% predicted and changes in neutrophil elastase.

Relationships between Sputum Biomarkers and Pulmonary Exacerbations

Examining the associations between sputum biomarkers (at baseline and changes over time) and the number of intravenous antibiotic courses showed that none of the baseline sputum biomarker measurements correlated with the number of antibiotic courses over the study period. However, declines in NEAPC and IL-6 were associated with a greater number of intravenous antibiotic courses (Table E5). Further data can be found in the online supplement.

Evaluation of a Single Determination of Sputum Neutrophil Elastase in the Validation Cohort

The correlations between the baseline neutrophil elastase (log-transformed) and annual change in FEV₁% predicted for the validation cohort are shown in Figure E2. The corresponding slope estimate for this relationship in the validation cohort (-1.07 [SE 0.82]) is very similar to the slope estimate for the main study cohort (-1.12 [SE 0.5]). To examine the predictive ability of sputum neutrophil elastase values for being a “rapid decliner” in the validation cohort, an FEV₁% predicted annual change cutoff of -2% predicted per year was used. Using this cutoff, 5 of the 10 validation cohort subjects were considered “rapid decliners,” and all but one subject had increased neutrophil elastase (log-transformed) compared with the “slow decliners,” resulting in good discriminative ability between these groups ($c = 0.76$). The one subject with a high baseline neutrophil elastase value who

experienced no decline in FEV₁% predicted was 20 years old, which is outside the age range of the main study cohort.

DISCUSSION

In this study of school-age children with CF, concentrations of sputum biomarkers of inflammation change over time, a single determination of sputum biomarkers, has predictive value for subsequent decline in FEV₁, and changes in sputum biomarkers are related to changes in FEV₁. Neutrophil elastase, TIMP-1, and TNF- α increased over time, whereas NEAPC and SLPI significantly decreased over the study period. Higher detectable baseline neutrophil elastase concentrations were associated with a faster decline in FEV₁. When categorizing subjects as “rapid decliners” or “slow decliners,” the initial measurement of neutrophil elastase had the highest individual predictive value

Figure 2. Receiver operating characteristic curves of baseline sputum biomarker combinations and FEV₁ decline. The initial measurement of neutrophil elastase had the highest individual predictive value, and neutrophil elastase, IL-8, and IL-6 had the highest combined predictive value for predicting a rapid decliner, defined as a rate of decline in FEV₁ greater than 2% per year. A perfect test is indicated by an area under curve value of 1. A test with no discriminatory value has an area under curve of 0.50 (diagonal line).

TABLE 5. CORRELATIONS BETWEEN LONGITUDINAL CHANGES IN FEV₁ AND CHANGES IN SPUTUM BIOMARKERS

Biomarker	<i>r</i> (P Value)
Total neutrophil count, log	−0.42 (0.01)
Neutrophils, %	−0.27 (0.12)
Elastase log, µg/ml	−0.55 (<0.01)
MMP-9 log, ng/ml	−0.22 (0.21)
NEAPC log, ng/ml	0.32 (0.07)
SLPI log, ng/ml	0.32 (0.06)
TIMP-1 log, ng/ml	−0.13 (0.47)
IL-1β log, pg/ml	−0.43 (0.01)
IL-6 log, pg/ml	−0.09 (0.62)
IL-8 log, pg/ml	−0.32 (0.06)
TNF-α log, pg/ml	−0.23 (0.19)

Definition of abbreviations: MMP-9 = matrix metalloproteinase 9; NEAPC = neutrophil elastase antiprotease complex; SLPI = secretory leukoprotease inhibitor; TIMP-1 = tissue inhibitor of metalloproteinase 1.

Bold values indicate statistically significant correlations.

for subsequent lung function decline, whereas neutrophil elastase, IL-8, and IL-6 had the highest combined predictive value of any three biomarkers. Furthermore, we found that a decline in FEV₁% predicted was significantly associated with increases in neutrophil counts, neutrophil elastase, and IL-1β and marginally associated with declines in NEAPC and SLPI and rising IL-8. These results support our hypothesis that changes in sputum biomarkers of inflammation, and proteolytic biomarkers in particular, are related to changes in lung function. Infection with *P. aeruginosa* and other bacteria did not predict or explain lung function decline in our study cohort.

There is a paucity of data in the literature examining the relationships between longitudinal changes in airway inflammation and lung function in CF. In a retrospective review of sputum biomarkers measured in multiple CF clinical trials, longitudinal analyses revealed significant associations between increases in neutrophil elastase and decreases in FEV₁ among the subset of subjects with CF who were randomized to placebo (12). No longitudinal correlations were observed with sputum neutrophil counts and IL-8. Although the Mayer-Hamblett study (12) relied on spontaneously expectorated sputum samples (not induced) collected mainly from adolescents and adults with more advanced lung disease than the subjects in our study cohort, their results are reasonably comparable to our study findings in terms of concentrations of sputum inflammatory mediators and the outcome of a longitudinal association between FEV₁ and neutrophil elastase. In a single-center study of older subjects with CF with worse lung function than that in our study cohort, changes in sputum DNA concentrations (but not IL-8 or myeloperoxidase concentrations) at two discrete time points within 1 year correlated with changes in lung function (21). Another study that used bronchoalveolar lavage (BAL) rather than sputum to investigate the long-term effect of recombinant human DNase on inflammation in a study cohort similar to ours (mainly children with CF with mild lung disease), reported a significant increase in BAL biomarkers of airway inflammation (neutrophil counts, free neutrophil elastase, and IL-8) in untreated subjects, whereas levels of these biomarkers remained relatively unchanged in patients treated with DNase (22). In this same study, patients with a normal percentage of neutrophils in BAL fluid at baseline did not show a decline in lung function over a 3-year period, a finding supporting the view that airway inflammation has a negative impact on subsequent lung disease progression in CF.

Lung function decline is a key clinical issue in CF. Being able to use biomarkers to monitor disease progression, and possibly even to predict those subjects at risk of experiencing more rapid decline in lung function, would have a significant impact on clinical

practice in CF. Our findings suggest that sputum inflammatory biomarkers, particularly protease and antiprotease measures, have predictive value for subsequent lung function decline, and changes in sputum biomarkers are related to changes in FEV₁. This study represents an important step in the validation of sputum inflammatory biomarkers as correlates of disease activity and progression. The next steps will be to establish cut-off points for individual sputum biomarkers that separate high versus low values for the CF population and determine the “minimal clinically meaningful difference” in sputum biomarker measurements that corresponds to a change in clinical status.

The results from this study provide justification for interventions that neutralize free neutrophil elastase activity or augment local antiprotease levels to counteract lung function decline in CF. It is likely that these antiinflammatory therapies will be most effective in patients with higher burdens of protease activity in their airways. In fact, exogenous administration of antiproteases to supplement levels in the CF airways has been under investigation since 1990 (23). A few more recent studies of α₁-antitrypsin agents demonstrated modest reductions or no significant increase in sputum neutrophil elastase activity (24–27). There has been renewed interest in the pharmaceutical industry to develop and test α₁-antitrypsin therapies in CF, and clinical trials are once again being planned. In fact, we are able to derive sample size calculations for such trials based on a standard deviation of 0.5 for the change in log neutrophil elastase over time that was observed in our study cohort. This estimate of variability is very similar to that previously reported (12). Eighteen subjects in each group would be required to detect a difference in means of 0.5 log neutrophil elastase between two randomized groups, and 49 patients per group would be required to detect a 0.3 log difference in means, assuming an α level of 0.05 and 80% power.

The limitations of this study need to be considered. This was a single-center study that enrolled children who mostly had normal or mildly abnormal lung function. Thus, our findings may not apply to adults or patients with CF who have more severe lung disease. A possibility of selection bias exists. We randomly and consecutively recruited children from our clinics and did not target specific individuals for enrollment. The rate of decline in FEV₁ in our cohort closely approximated the rate observed in all school-age children with CF followed at our center and is reasonably comparable to that reported in other recent studies of children and adolescents with CF (13, 22, 28, 29). This suggests that our small study cohort relatively closely reflects the larger population of school-age children with CF. Our finding that a higher baseline FEV₁ is associated with faster rates of subsequent FEV₁ decline is consistent with previous studies (13, 30). In contrast, although we found male gender to be a risk factor for faster decline in FEV₁ among our cohort, most previous studies report that female gender is the more significant risk factor (13, 28, 30, 31). In addition to the independent effects of age, gender, and starting FEV₁ on lung function decline, we show that children in our study with higher levels of neutrophil elastase tend to experience more rapid decline in FEV₁ over time. These associations remained significant even after adjusting for age, gender, and baseline FEV₁, indicating that airway inflammation plays a role in lung function decline.

Although we intended to study subjects during times of relative clinical stability (as defined in MATERIALS AND METHODS section), frequently, subjects had increased symptoms at the times of these study visits that were not severe enough to warrant hospitalization but often resulted in a new treatment or management approach. Additionally, although this was an observational study, results from lung function testing and sputum inductions performed during study visits led to a change in treatment. In fact, CF-related pathogens, including *P. aeruginosa*, *Stenotrophomonas*

Figure 3. Associations between longitudinal changes in FEV₁ and changes in select sputum biomarkers.

maltoophilia, and *Aspergillus*, were identified more frequently in induced sputum cultures collected during study visits when compared with the nearest respiratory cultures within 4 months of these study visits, resulting in a new antimicrobial prescription at 6% of study visits (32). This is consistent with recent data that induced sputum has a higher microbiological yield compared with conventional respiratory samples in children with CF, even in subjects capable of expectorating sputum spontaneously (33). Despite these limitations, we found significant changes in sputum biomarkers that related to changes in lung function.

There are a number of inflammatory markers or mediators that have been or could be measured in sputum (34). We focused on a panel of proteases, antiproteases, and cytokines because of their purported role in airway inflammation and supporting data in the CF literature and because we hypothesized that a change in airway proteolytic activity would relate to changes in lung function. Future studies need to consider other candidate biomarkers, such as high mobility group box protein-1 (35) or calprotectin (36), or explore the effects of other potential disease-modifying pathways, such as hormonal changes, which have been implicated in CF disease progression (37). It will also be important to compare the relative value of systemic biomarkers measured in blood and urine to local biomarkers assessed in sputum for monitoring CF disease activity and progression.

In summary, longitudinal changes in sputum biomarkers of inflammation relate to lung function changes, and determinations of sputum biomarkers have predictive value for subsequent lung function decline among a cohort of school-age children with CF. Based on our results, sputum neutrophil elastase, implicated in disease pathogenesis and in the development of bronchiectasis, appears to be the most informative individual biomarker to monitor disease activity and represents a feasible treatment target in CF.

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