Proteomic Analysis of Sputum from Adults and Children with Cystic Fibrosis and from Control Subjects

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Rationale: **Recurrent pulmonary exacerbations are associated with progressive lung disease in cystic fibrosis (CF). Current definitions of an exacerbation, although not precisely defined, include new/worsening symptoms, declining lung function, and/or changing radiologic appearance. Early diagnosis of exacerbations by rapid noninvasive means should expedite therapeutic intervention, thereby minimizing lung damage.**

Objectives: **To identify biomarkers of lung exacerbation for point-of-care monitoring of CF lung disease progression.**

Methods: **Saline-induced sputum was collected from adults with CF with an exacerbation and requiring hospitalization ($FEV_1 < 60\%$), a subset of these adults at hospital discharge, children with stable CF and preserved lung function ($FEV_1 > 70\%$), and control subjects ($FEV_1 > 80\%$). Sputum was arrayed by two-dimensional electrophoresis and differentially expressed proteins were identified by proteomic analysis.**

Measurements and Main Results: **Sputum profiles from adults with CF with an exacerbation were characterized by extensive proteolytic degradation and influx of inflammation-related proteins, with some adults with CF approaching a "healthy" protein profile after hospitalization. Two children with CF showed profiles and biomarker expression resembling those of adults with an exacerbation. Levels of differentially expressed myeloperoxidase, cleaved α_1-antitrypsin, IgG degradation, interleukin-8, and total protein concentration, together with their correlation to FEV_1, were statistically significant. Statistical correlation analyses indicated that changes in myeloperoxidase expression and IgG degradation were the strongest predictors of FEV_1.**

Conclusions: **We identified extensive protein degradation and differentially expressed proteins as biomarkers of inflammation relating to pulmonary exacerbations. Prediction of exacerbation onset and more precise evaluation of the extent of resolution with treatment could be achieved by including biomarkers in standard assessment.**

Keywords: α_1-antitrypsin; exacerbation; immunoglobulin; inflammation; myeloperoxidase

Cystic fibrosis (CF) is one of the most common lethal genetic diseases in white individuals, with a carrier rate of approximately 4 to 5% and an incidence of approximately 1 in 2,500. CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) (1). Symptoms include salty-tasting skin; persistent coughing, sometimes with phlegm; wheezing; shortness of breath; malnutrition; abdominal pain; and frequent, bulky, greasy stools (1). Symptoms vary between individuals, partly due to more than 1,000 known mutations of the CFTR gene. The ΔF508 mutation accounts for approximately 70% of the CFTR mutations in white populations (2, 3).

Progressive lung disease is the major cause of morbidity and mortality in patients with CF (1). Airways become colonized with bacteria, particularly *Pseudomonas aeruginosa* and *Staphylococcus aureus*, while recurrent pulmonary infections and inflammation result in submucosal gland hypertrophy and excessive mucous secretion in the lungs (4, 5). Impaired mucociliary clearance and plugging of small airways cause progressive bronchiectasis, ultimately resulting in respiratory failure (6, 7). Lung disease starts as early as the first few months of life and is difficult to detect without invasive techniques such as flexible bronchoscopy and bronchoalveolar lavage (8). Although CF can be diagnosed in newborns by genetic screening (9), therapy is directed by evaluation, which includes review of symptoms, lung function, and to a lesser extent radiologic changes, and is therefore likely to lag behind the occurrence of established lung pathology.

Proteins are the ultimate product of gene expression and the development of prognostic tests and drugs for CF will occur through a greater understanding of the proteins and their interactions within the lung environment. Proteomics provides the ability to characterize proteins and their post-translational modifications and offers a greater understanding of the physiology of the lung environment. For complex solutions, such as sputum, two-dimensional (2-D) electrophoresis represents a key technology of choice for arraying and characterizing constituent proteins, and has been used to help characterize protein expression in bronchoalveolar lavage fluid (BALF) from individuals with CF (10, 11). This study, which represents the first proteomic study to address differential sputum proteomes in the context of subjects with CF versus control individuals, aims to identify protein biomarkers that are indicative of an acute pulmonary exacerbation. Sputum protein profiles from subjects with CF with an exacerbation have been compared with those from a subgroup of the same subjects with CF after hospital treatment, with clinically stable children with CF with preserved lung function, and with control subjects to further elucidate changes in protein profiles and expression as markers of disease progression.

Understanding changes in protein expression with pulmonary disease will permit development of clinical assays for rapid, noninvasive analysis of fluids such as blood, sputum, or saliva. This will help elicit early intervention of severe airway infection and/or acute inflammatory responses and help dictate short- and long-term therapy for CF lung disease. Minimizing cumulative pulmonary deterioration from the recurring cycle of infection and inflammation will ultimately help prolong the length and improve the quality of life for an individual with CF (5, 12).

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METHODS

Clinical Samples

Saline-induced sputum from human subjects was collected according to previously described methods (13, 14) from healthy control adults (18–40 yr of age; n = 20) and children (8–14 yr of age; n = 5) with forced expiratory volume in 1 s (FEV_1, %pred) greater than 80%; adults with CF and an acute pulmonary exacerbation (15, 16), FEV_1 less than 60%, and requiring hospitalization (n = 20); a subset of these adults with CF also at the time of hospital discharge (n = 13); and children with CF without clinical evidence of an exacerbation (n = 7), FEV_1 greater than 70% (Table 1). Sputum was immediately placed on ice and then solubilized within 1 h of collection. Sputum was solubilized for 1 h at 4°C, using methods similar to those previously described (13, 14). A complete ethylenediaminetetraacetic acid–free protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Mannheim, Germany) was added to sputum samples to prevent proteolytic degradation during solubilization. Cell and bacterial debris was subsequently removed by centrifugation at 2,000 × *g* (10 min at 4°C) and 0.2-μm pore size filtration before proteomic analysis. An identical clinical protocol was used for collection of samples from adults and children. On-site training ensured standardization of procedures at all sites of sample preparation. Sputum was qualified using a criterion of a squamous cell count less than 80% (14). Subjects with CF were excluded from this study if they had any other coexisting acute or chronic illnesses. Institutional human research ethics committees approved human subject recruitment and research involving human samples for these studies. Written consent was obtained from all subjects (or their legal guardians) participating in this study. These studies were conducted in accordance with the World Medical Association Declaration of Helsinki regarding ethical principles for medical research involving human subjects.

Sample Preparation and 2-D Gel Electrophoresis

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. After liquefaction, sputum proteins were resuspended in ProteomIQ CHAPS resuspension reagent (Proteome Systems, Inc., Woburn, MA) with 40 mM TRIS and reduced and alkylated with 5 mM tributylphosphine and 10 mM acrylamide for 1 h at room temperature. Samples were then desalted to remove TRIS and subsequently analyzed by 2-D electrophoresis (2-DE), using 11-cm IPG strips, pH 4–7, with either 6–15 or 14% polyacrylamide GelChIP gels (Proteome Systems Ltd, Sydney, Australia) using IsoelectrIQ[2] and ElectrophoretIQ[3] devices with a ProteomIQ platform (Proteome Systems Ltd) as previously described (17). Proteins (300 μg/gel) were visualized by staining gels with both SYPRO Ruby (Molecular Probes/Invitrogen, Eugene, OR) and Coomassie Brilliant Blue G-250. Differential protein expression was determined with ImagepIQ (Proteome Systems Ltd). Protein spots present in the majority of the CF adult (exacerbation) gels and whose expression levels showed distinct up- or downregulation when compared with respective gels at discharge and control subjects were selected for further analysis. Gel pieces were excised, destained, digested with trypsin, and desalted with Xcise (Proteome Systems Ltd and Shimadzu-Biotech, Kyoto, Japan) with a ProteomIQ Xcise in-gel digest kit (Proteome Systems, Inc.).

Mass Spectrometry

Protein digests were analyzed with an Axima-CFR matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (Kratos, Manchester, UK) as previously described (17). All solutions used for MALDI-TOF mass spectrometry analysis were from a ProteomIQ Xcise in-gel digest kit (Proteome Systems, Inc.). BioinformatIQ (Proteome Systems Ltd) was used for data analysis and tracking. Protein identifications were confirmed by nanoflow liquid chromatography–mass spectrometry with an LCQ DECA ion trap mass spectrometer (Thermo Electron Corp., Waltham, MA; *see* the online supplement for further detail).

ELISA and Western Blotting

ELISA. Myeloperoxidase levels in liquefied sputum were measured by sandwich ELISA using the 2C7 anti-human myeloperoxidase monoclonal antibody (Serotec, Oxford, UK). A rabbit anti-human myeloperoxidase polyclonal antibody (Chemicon International, Inc., Temecula, CA) and a sheep anti-rabbit horseradish peroxidase–conjugated antibody (Chemicon International) were used for detection. Interleukin-8 (IL-8) levels were measured with an IL-8 EASIA kit (BioSource, Nivelles, Belgium). *See* the online supplement for further detail.

Western blotting. Subsequent to 1-DE, sputum was analyzed by Western blotting with a sheep anti-mouse horseradish peroxidase–conjugated antibody (Chemicon International) to measure IgG expression (*see* the online supplement for further detail).

Statistical Analyses

Incidences and spot volumes of each protein in control and CF groups, determined by image analysis, were compared by Fisher's exact test and Student *t* test, respectively. The Student *t* test was based on two-way analysis of variance, which included terms for disease status, age (adult vs. child), and their interaction. Strong control of the family-wise type I error rate for each term (age, disease status, and interaction) was maintained by Holm's adjustment (18), applied to all the protein spot volumes. This procedure is a stochastically dominant modification of the Bonferroni procedure for testing each hypothesis at a p value of 0.05/K, where K is the number of protein spots analyzed. Statistical analyses were performed with S-Plus 6 software (Insightful Corporation, Seattle, WA).

Differences between biomarker concentration and disease groups. Data were analyzed with a linear model, applied separately for children and for adults. The linear model for children was a fixed effects linear model, with a term representing disease status. The mixed model was fitted with the residual maximum likelihood algorithm (19) as implemented in the R package nlme (20) (*see* the online supplement for further detail).

Relationships between biomarker concentration and lung function. The relationship of each biomarker with FEV_1 was assessed by two statistical tests.

Ignoring Other Markers: The effect of each marker was tested by looking at the reduction in sums of squares obtained when the marker is added to a model containing only the age effect.

Eliminating Other Markers: The effect of each marker was tested by looking at the reduction in sums of squares obtained when the marker is added to a model containing age and all the other markers. In addition, Akaike's information criterion was used in backward elimination, to select variables for inclusion in a final model (*see* the online supplement for further detail).

RESULTS

2-D Profiling of Sputum

Extensive differences in sputum protein profiles between adults with CF with an exacerbation and control subjects were observed (Figure 1). Destruction of whole proteins resulting from proteolytic degradation in sputum from adults with CF with an exacerbation was evidenced by the relative increase in low-molecular-weight protein. Disappearance of trains of protein spots was consistently observed in these 2-D profiles compared with control subjects. An influx of neutrophil-derived proteins, identified by mass spectrometry, into the sputum of subjects with CF with an exacerbation was also apparent (Figures 1C–1F). The number of protein spots observed in the 2-D profiles was different for each of the subject groups analyzed. The average number of spots for each group (mean ± SD) was as follows: adults with CF with exacerbation, 478 ± 124; adults with CF at discharge, 507 ± 101; control adults, 338 ± 64; children with CF, 441 ± 75; control children, 382 ± 51.

Sputum from 13 of the 20 adults with CF was also collected at hospital discharge, approximately 2 wk after admission. Sputum profiles from four of these discharged adults with CF, particularly subject 11 (Figure 1D), resembled those of control subjects (Table 1). Profiles from eight discharged adults with CF did not show clear evidence of proteomic improvement despite an increase in FEV_1 (Table 1). Interestingly, CF subject 39 had an improvement

TABLE 1. CLINICAL AND PROTEOMIC CHARACTERISTICS OF CYSTIC FIBROSIS AND CONTROL ADULT AND CHILD SUBJECTS

Subject No.	Sex	CFTR Mutation	Age (*yr*)	BMI (kg/m^2)		Baseline FEV_1 (*%pred*)		Sputum Protein Concentration (*mg/ml*)		Discharge Sputum 2-DE Profile Status*
				Exac.	Dis.	Exac.	Dis.	Exac.	Dis.	
Adults with CF										
4	M	ΔF508/ΔF508	28	23.3		52.3		1.15		—
6	F	ND	ND	ND	ND	ND	ND	1.34		—
10	M	ΔF508/ND	26	15.7		17.8		0.36		—
11	F	ΔF508/ΔF508	24	20.3	20.3	59.5	81.3	2.275	0.22	↑↑↑
12	M	ΔF508/ΔF508	37	23.1	24.2	53.5	62.3	1.62	0.82	NC
15	M	ΔF508/ΔF508	21	26.6		50.4		1.45		—
16	F	ΔF508/ΔF508	21	21.3	21.6	46.7	46.0	1.33	0.78	NC
20	F	ΔF508/G551D	31	21.3	22.4	21.9	35.4	0.94	0.59	↑
35	F	ΔF508/ΔF508	32	21.5		59.1		1.29		—
37	M	ΔF508/ND	31	27.5	26.6	47.8	40.6	0.63	1.839	↓
38	F	ΔF508/G542X	20	17.7	17.7	14.3	27.3	0.499	0.773	NC
39	F	ΔF508/ΔF508	35	20.7	20.9	66.9	82.7	0.774	2.625	NC
40	F	ΔF508/ΔF508	19	15.5	15.5	18.4	25.8	1.666	1.834	NC
41	F	ΔF508/ΔF508	28	20.6	20.6	60.7	67.2	0.412	0.21	↑↑↑
43	M	ΔF508/G542X	24	17.9	19.7	46.4	69.4	1.353	1.364	NC
44	F	ΔF508/R560T	19	18.2	19.1	56.9	76.0	0.675	0.425	↑↑
45	F	ΔF508/ΔF508	19	17.6	18.4	30.7	26.7	0.997	0.675	NC
46	F	ΔF508/ΔF508	21	18.6	18.5	55.6	60.5	1.02	0.28	NC
47	F	ΔF508/G542X	38	18.6		39.4		0.695		—
48	F	ND	ND	ND		ND		0.863		—
Children with CF										
61	F	ΔF508/ΔF508	12	19.8		102		0.213		—
63	M	ΔF508/W1282X	12	18.8		97		0.421		—
64	M	ΔF508/ΔF508	13	17.1		97		0.656		—
65	F	ΔF508/W1282X	12	18.0		80		0.291		—
69	F	ΔF508/ΔF508	11	21.8		86		1.191		—
71	M	ΔF508/ND	10	17.5		101		0.201		—
76	M	ΔF508/ΔF508	12	20.4		73		0.401		—
Control adult subjects										
7	M	—	26	20.1		90.2		0.23		—
8	F	—	21	24.7		96.3		0.36		—
9	F	—	25	26.1		98.7		0.064		—
13	M	—	23	24		94.1		0.202		—
17	M	—	32	27.1		114.5		0.09		—
18	M	—	20	26.3		122.4		0.1		—
19	F	—	39	18.4		103.5		0.09		—
21	F	—	23	20.7		98.3		0.22		—
22	F	—	22	22.0		116.2		0.16		—
23	F	—	20	23.1		93.6		0.188		—
24	F	—	19	18.1		102.2		0.059		—
25	F	—	33	20.8		92.2		0.085		—
26	F	—	21	20.8		105.1		0.15		—
27	F	—	23	21.4		108.8		0.2		—
28	F	—	20	23.7		102.7		0.074		—
29	M	—	21	26.0		110.2		0.055		—
30	M	—	30	23.0		107.5		0.09		—
32	F	—	31	22.6		86.6		0.073		—
34	F	—	31	22.8		94.6		0.063		—
36	M	—	32	22.0		98.2		0.096		—
Control child subjects										
70	F	—	10	21.8		89		0.238		—
72	M	—	9	16.9		97		0.229		—
73	F	—	10	17.2		123		0.45		—
75	F	—	10	16.2		95		ND		—
79	M	—	12	16.0		96		0.322		—

Definition of abbreviations: 2-DE = two-dimensional electrophoresis; BMI = body mass index; CF = cystic fibrosis; CFTR = cystic fibrosis transmembrane conductance regulator; Dis. = discharge; Exac. = exacerbation; F = female; M = male; NC = no change; ND = not determined.

Subjects were specifically recruited for this study, and subject number allocation was based on order of recruitment, irrespective of CF or control status. Subject numbers 60 and below correspond to adult subjects, whereas subject numbers greater than 60 correspond to child subjects. Missing subject numbers correspond to subjects who were excluded from this study as a result of not satisfying inclusion criteria. Sputum from these excluded subjects was not analyzed in this proteomic study. Values for BMI, FEV_1, and total sputum protein concentration for adults with CF both at hospital admission when presenting with an acute exacerbation (Exac.) and at discharge (Dis.) are shown in respective columns. A proteomic improvement (↑) was deemed to be shown by a change in the sputum 2-DE profile toward one more closely resembling that of a control subject. Sputum total protein concentration was determined after liquefaction, using a Bradford protein assay (Sigma-Aldrich, St. Louis, MO).

* On the basis of how well a profile approaches that of a control.

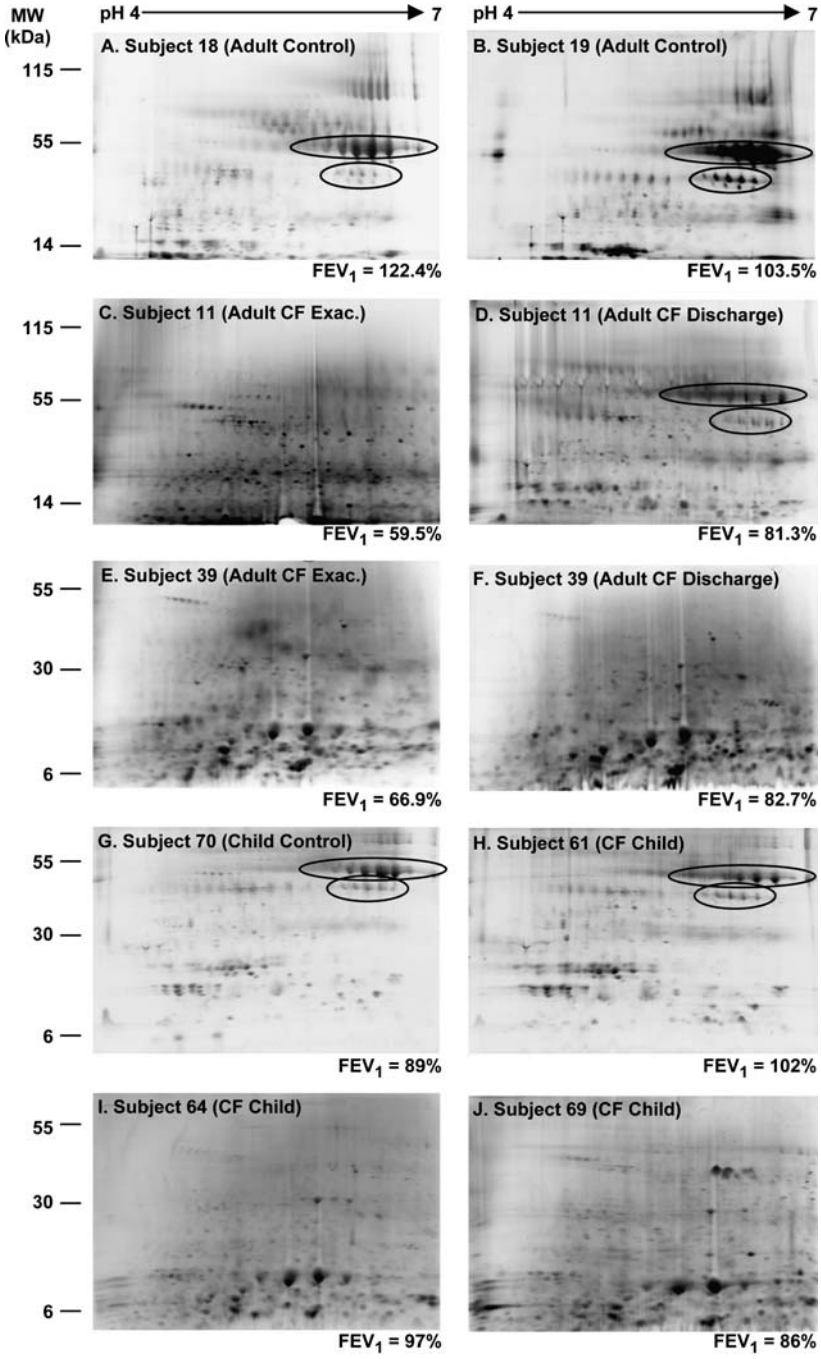

Figure 1. Representative two-dimensional electrophoresis (2-DE) protein profiles of sputum from adult and child subjects with cystic fibrosis (CF) and adult and child control subjects. The 2-DE protein profiles of sputum are shown for adult control subjects 18 (*A*) and 19 (*B*), adult CF subject 11 at exacerbation (*C*) and at hospital discharge (*D*), adult CF subject 39 at exacerbation (*E*) and at hospital discharge (*F*), child control subject 70 (*G*), child CF subject 61 (*H*), child CF subject 64 (*I*), and child CF subject 69 (*J*). Sputum samples were analyzed over a pH 4–7 range, using 6–15% (wt/vol; *A–D*) and 14% (wt/vol; *E–J*) polyacrylamide GelChIP gels. Trains of proteins are clearly visible (examples have been *circled*) in profiles of all control subjects and subjects with CF who appear to be in a nonexacerbated state. Of note are the two sputum profiles for child CF subjects 64 and 69, which are more characteristic of an adult with CF with an exacerbation, that is, proteolytic degradation is evidenced by the increase in lower molecular weight proteins and an influx of neutrophil-derived proteins. *C* and *D* compared with *E* and *F* provide an example of an individual (subject 11) with clinical improvement as assessed by FEV_1 with a corresponding "normalization" of sputum proteomic signature at discharge as compared with an individual (subject 39) with clinical improvement as assessed by FEV_1 but no normalization of sputum proteomic signature at discharge.

in FEV_1 from 66.9 to 82.7% at discharge, yet displayed minimal change in sputum profile (Figures 1E and 1F); the remaining inflammation-derived proteins, which will continue to cause tissue damage, suggests poor lung recovery from the recent exacerbation. Adult CF subject 37 was diagnosed with a viral infection 3 d before discharge. This was reflected by a decline in respiratory status, determined by FEV_1 and sputum profiling, which showed increased levels of protein degradation and neutrophil-derived proteins.

Clinical criteria were used to define children with CF with stable disease and preserved lung function as a second control group for the adults with CF with an exacerbation. Despite all children with CF having clinical measures, particularly FEV_1, similar to those of control subjects (Table 1), CF subjects 64 and 69 presented sputum profiles clearly indicating signs of inflammation as observed for adults with CF with an exacerbation (Figures 1I–1J). Thus, although FEV_1 measurements indicated healthy respiratory capacity, proteomic data indicated early signs of inflammation and/or infection. Interestingly, CF Child 64 was clinically diagnosed as having a flare of allergic bronchopulmonary aspergillosis 96 d after proteomic analysis. An elevation of total IgE associated with a drop in lung function, new infiltrates, and acute respiratory signs were found at the time of the flare. Child CF subject 69 was clinically diagnosed 49 d later with a flare of *S. aureus* infection. The other children with CF remained stable during these time frames.

Biomarker Identification

We identified a number of neutrophil-derived and inflammation-associated proteins that were differentially expressed between

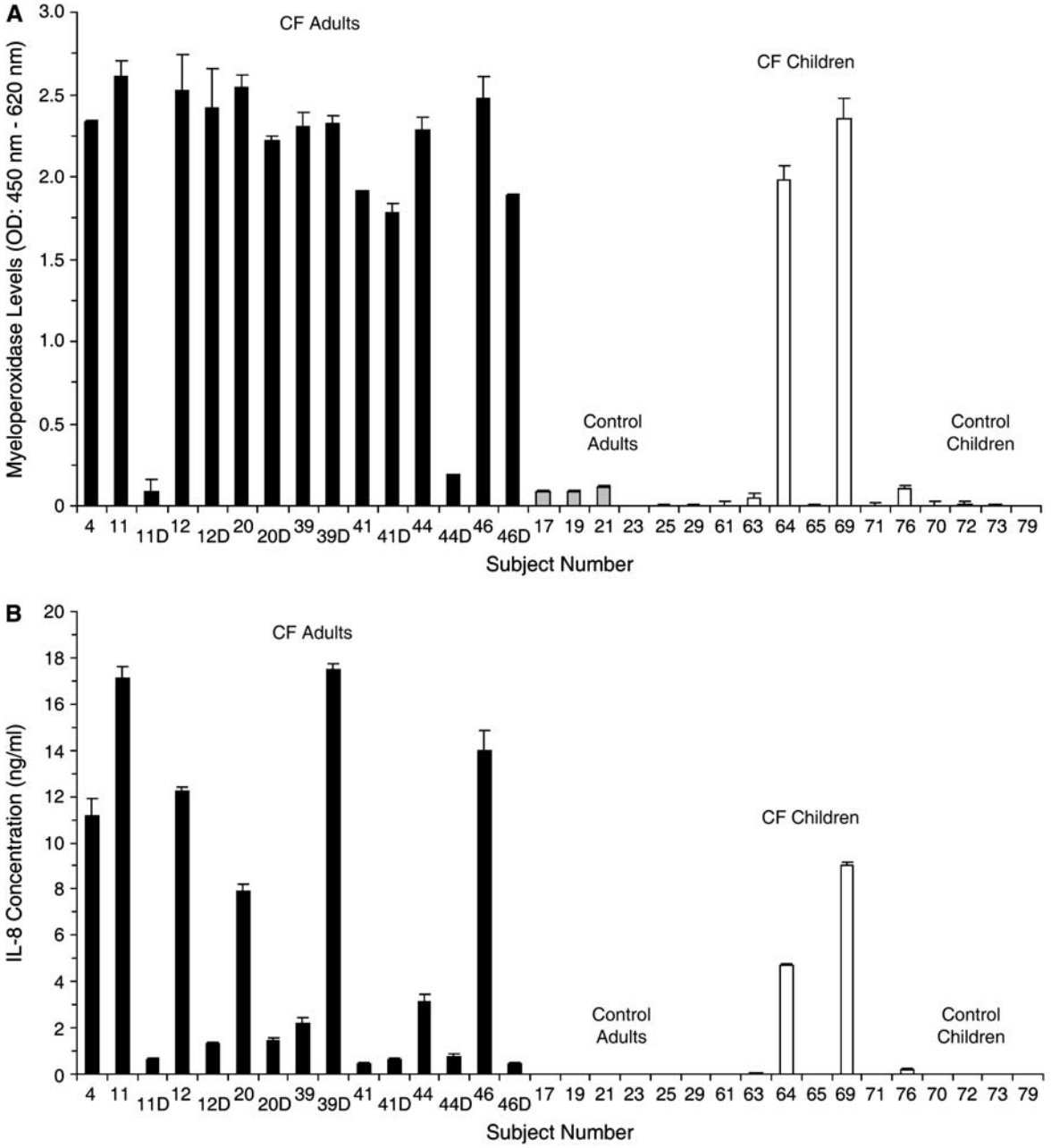

Figure 2. Relative myeloperoxidase levels (*A*) and interleukin (IL)-8 concentrations (*B*) in sputum from subjects with CF and control subjects. Myeloperoxidase levels and IL-8 concentrations were measured by ELISA in adults with CF with an exacerbation and a subset of these adults with CF at hospital discharge (indicated by a "D" in subject ID; *solid columns*); and in control adults (*light gray columns*); children with CF (*open columns*); and control children (*dark gray columns*). Samples were analyzed in duplicate, and *error bars* indicate the standard deviation. Myeloperoxidase standards were not available and therefore levels were measured by optical density (OD). For myeloperoxidase measurements, the mean absorbance for control subjects was subtracted from all data points, whereas for IL-8 measurements background absorbance levels corresponding to a 0-pg/ml standard were subtracted from all data points.

adults with CF with an exacerbation and control subjects at statistically significant levels ($p \leq 0.005$), using 2-DE–based proteomics. These differences were also observed in child CF subjects 64 and 69 relative to control subjects. We have characterized three of these proteins, myeloperoxidase, α_1-antitrypsin, and IgG, for this study.

Differential expression of myeloperoxidase in sputum between CF and control subjects was confirmed by ELISA (Figure 2A). Mean absorbance ($\pm$ SD) for myeloperoxidase levels in adults with CF with an exacerbation was 2.74 ± 0.22 AU compared with 0.37 ± 0.10 AU in control adults. Adult CF subjects 11, 44, and 46 showed a significant decrease in expression of myeloperoxidase after hospitalization, whereas other adults with CF showed minimal change. These patterns closely support clinical data and observations made by 2-D profiling of sputum (Table 1). In contrast, discharged adult CF subject 39, with clinical recovery from an exacerbation, showed no change whereas CF child subjects 64 and 69, meeting the definition of stable disease, showed

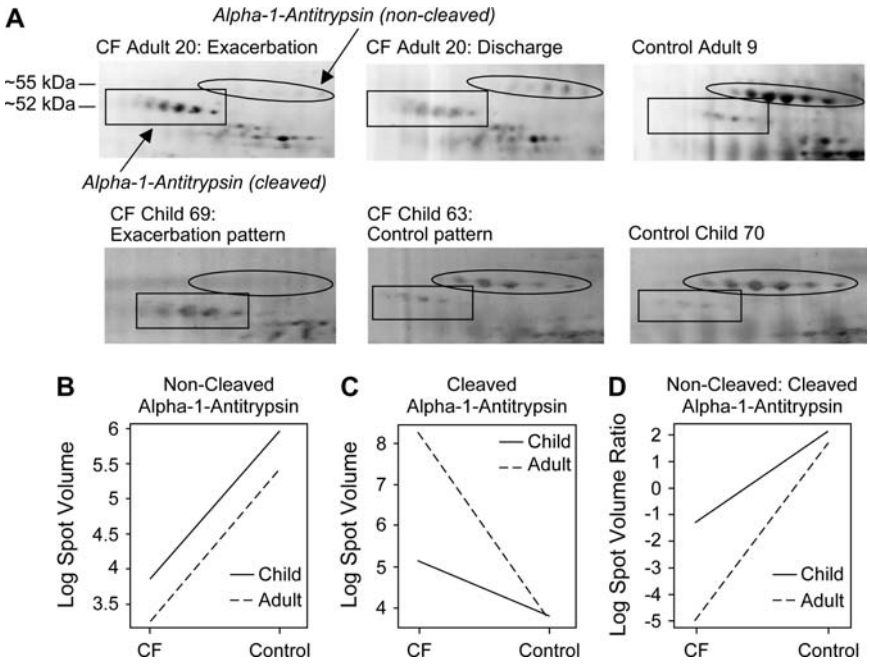

Figure 3. Differential expression of α_1-antitrypsin protein isoforms in sputum from CF and control adult and child subjects and the statistical relationship between subject status and expression levels of noncleaved, cleaved, or the ratio of noncleaved to cleaved, α_1-antitrypsin. (*A*) Various representative gel images showing differential expression of cleaved (*rectangle*) and noncleaved (*oval*) isoforms of α_1-antitrypsin in sputum between adult CF subject 20 during an exacerbation and at hospital discharge; adult control subject 9; child CF subject 69, who presented a 2-D sputum profile resembling that of a CF adult with an exacerbation; child CF subject 63, who presented a 2-D sputum profile resembling that of a control; and control child subject 70. Log-transformed normalized gel spot volumes, the latter determined with ImagepIQ image analysis software (Proteome Systems Ltd), were analyzed for (*B*) noncleaved α_1-antitrypsin, (*C*) cleaved α_1-antitrypsin, and (*D*) the ratio of noncleaved to cleaved variants of α_1-antitrypsin. Data were analyzed by two-way analysis of variance, with effects for subject status (CF or control), age (adult or child), and subject CF/control status by age interaction analyzed. The interaction plots in *B* and *C* indicate that CF/control status can be distinguished because control adults and children have lower levels of noncleaved and/or cleaved α_1-antitrypsin compared with adults with CF and children with CF, who have significantly higher levels. Analyzing cleaved levels of α_1-antitrypsin illustrates the difference in CF disease status effect between adults with CF with an exacerbation and children with CF with stable disease and preserved lung function (*C*). A similar disease status effect is shown for the ratio of noncleaved to cleaved levels of α_1-antitrypsin (*D*). In this case a reciprocal relationship is observed, given the ratio response is driven by the cleaved form of α_1-antitrypsin.

relative increased levels of myeloperoxidase (Figure 2A). As a known marker of inflammation (21), IL-8 concentrations in sputum were measured by ELISA as a comparison (Figure 2B). Expression patterns of myeloperoxidase versus IL-8 were found to be similar for the respective children. For the adults, other than CF subjects 39 and 41, there was a clear drop in IL-8 for subjects with CF at discharge relative to the exacerbation time point. Interestingly, the change in expression of IL-8 for CF subjects 12, 20, and 46 was greater for IL-8 than for myeloperoxidase, yet for CF subject 44 the change in myeloperoxidase expression was greater at discharge. For CF subject 41, IL-8 concentration was close to that of control subjects, despite 2-DE profiles and myeloperoxidase levels clearly indicating extensive inflammation. IL-8 concentration in sputum from CF subject 39 more closely mirrored the profile observed by 2-DE, which worsened at discharge, despite an improvement in FEV_1. These data suggest that these proteins may be markers of different, albeit related, stages of the inflammation process.

Protein spots corresponding to putative proteolytic cleavage products of α_1-antitrypsin were observed in adults with CF with an exacerbation and in CF Child subjects 64 and 69, as defined by a decrease in both pI ($\sim$ 0.5 units) and molecular mass ($\sim$ 3 kD; Figure 3A). Measurement of noncleaved α_1-antitrypsin showed a large disease status effect (CF vs. control) for adults and children ($p = 0.007$); however, there was a clear lack of age (adult vs. child) by CF/control status interaction (Figure 3B). In contrast, measurement of cleaved α_1-antitrypsin or the ratio of noncleaved to cleaved α_1-antitrypsin showed a clear CF versus control effect (both $p < 0.001$) as well as an age by CF/control status interaction that was also statistically significant ($p < 0.002$ and $p < 0.022$, respectively; Figures 3C and 3D). Adult and child control subjects had similar levels of cleaved α_1-antitrypsin, but adults with CF with an exacerbation had much higher levels than did children with CF. Normalized gel spot volumes of cleaved α_1-antitrypsin or the ratio of noncleaved to cleaved α_1-antitrypsin also distinguished between adult and child CF cohorts ($p < 0.002$ and $p < 0.006$, respectively; Figures 3C and 3D). Clearly the ratio response is driven by the cleaved α_1-antitrypsin response.

Degradation of Immunoglobulin in Children with CF

One-dimensional gel analysis of sputum was performed to determine the IgG profiles between CF and control adults and children. Western blotting and MALDI-TOF mass spectrometry analyses identified numerous IgG-γ_1 heavy-chain fragments in sputum, molecular mass about 25 to 45 kD, from all adults with CF with an exacerbation but only full-length chains in adult control subjects (Figure 4A). The patterns of degradation, quantified through calculation of a degradation ratio, closely mirrored the 2-D profiles at exacerbation and discharge described in Figure 1, where IgG degradation is clearly distinguishable from the lack thereof in control subjects. Subjects 11, 41, and 44 show a clearance of this degradation pattern at discharge. Similar analyses of sputum from the children revealed that the two children with CF with proteomic profiles similar to those of adults with an exacerbation (i.e., subjects 64 and 69) also expressed similar fragments of IgG heavy chain (Figure 4B). Sputum from all other children with CF and control children contained only full-length IgG heavy and light chains.

Statistical Analyses: Relationships between Disease Groups, Biomarker Expression, and Lung Function

Differences between disease groups. Combined analysis of expression of total protein concentration, myeloperoxidase, IL-8, IgG degradation, and cleaved α_1-antitrypsin all showed statistically significant differences between disease groups for the adults (Table 2). For each biomarker, values were smallest in control groups and largest in the CF exacerbation group. The values for adults with CF at discharge were in between those of the control

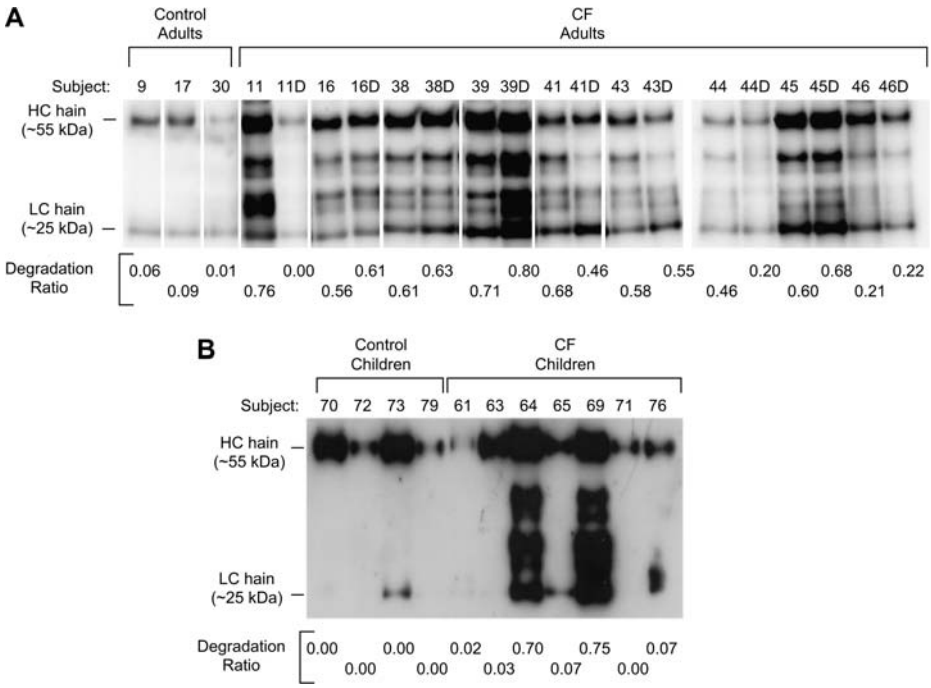

Figure 4. IgG degradation in sputum from adults with CF (*A*) and children (*B*). Liquefied sputum samples were analyzed by 1-DE and Western blotting, using a sheep anti-human IgG horseradish peroxidase–conjugated antibody. Full-length heavy (H) and light (L) chains of IgG are indicated. The degradation ratio represents the ratio between the sum of the intensities of the degraded heavy chain bands and the total sum of the intensities for all heavy chain bands, including the full-length heavy chain after background subtraction.

and CF exacerbation groups. For FEV_1, again the adults showed marked statistically significant differences between disease groups, with FEV_1 values for subjects with CF experiencing an exacerbation being the worst (smallest) and values for control subjects being the best (highest; Table 2). Differences for children, in biomarker concentrations or FEV_1, were not statistically significant, although they were almost so for IL-8 (Table 2).

Relationships between biomarker concentration and lung function. Tests for the relationship of each biomarker, including total sputum protein concentration, with FEV_1, both ignoring and eliminating the effects of the other markers, are shown in Table 3A. It is clear that each of the biomarkers is statistically significantly associated with FEV_1 (each of the biomarkers has statistically significant F values, ignoring the other markers). However, because of the strong correlation between biomarkers, no biomarker is statistically significant when added to a model that includes the other biomarkers. The implication is that at least one of the biomarkers may be used to predict FEV_1, but that the full set of biomarkers is not required to achieve this. When biomarkers were selected with Akaike's information criterion, only two proteins were included in the model: log myeloperoxidase optical density and IgG degradation. The other biomarkers (IL-8 and total protein concentration) were dropped. That is, the use of Akaike's information criterion suggested that myeloperoxidase concentration and IgG degradation have a stronger relationship with FEV_1 than does adult/child status, IL-8, or total protein concentration.

When a dataset containing matched α_1-antitrypsin data was further analyzed, each of the biomarkers was again statistically significant when considered while ignoring the effects of the others, but none were statistically significant when considered while eliminating the effect of the other markers (Table 3B). The strongest marginal relationship was between FEV_1 and the ratio of noncleaved to cleaved α_1-antitrypsin. However, backward elimination based on Akaike's information criterion for the data in Table 3B resulted in a model containing only log

TABLE 2. MEAN BIOMARKER EXPRESSION BY DISEASE GROUP

	Adults					Children			
		CF							
	Control	Discharge	Exacerbation	F Value	Pr(> F)	Control	CF	F value	Pr(> F)
Log protein concentration	−1.591	−0.320	0.086	28.635	0.000	−0.914	−0.667	0.789	0.398
Log IL-8 concentration	−3.604	0.324	1.737	41.232	0.000	−3.512	−1.062	4.664	0.059
Log myeloperoxidase concentration	−1.741	0.285	0.941	32.266	0.000	−1.610	−0.830	1.707	0.224
IgG degradation	0.052	0.462	0.574	7.116	0.005	−0.003	0.235	1.885	0.203
FEV_1	102.312	57.709	47.117	28.341	0.000	101.250	90.857	1.728	0.221
Log cleaved α_1-antitrypsin	2.849		8.461	41.211	0.000	3.578	4.636	0.732	0.402
Log ratio of noncleaved to cleaved α_1-antitrypsin	1.929		−5.982	25.433	0.000	2.347	−1.748	3.408	0.080

Definition of abbreviations: CF = cystic fibrosis; IL = interleukin; Pr(> F) = tail area probability of the F value.

Mean log concentrations of total sputum protein concentration (mg/ml), IL-8 concentration (ng/ml), and myeloperoxidase levels (optical density), cleaved α_1-antitrypsion (spot volumes) and the ratio of noncleaved to cleaved α_1-antitrypsin (spot volumes), as well as IgG degradation (band intensity) and mean FEV_1 (%pred) for corresponding subjects in each age and disease group are shown. F ratios and p values are for the hypotheses of no disease differences in biomarker concentration for adults and for children.

TABLE 3. RELATIONSHIP OF BIOMARKER EXPRESSION TO FEV_1

	Ignoring Others		Eliminating Others	
	F Value	Pr(> F)	F Value	Pr(> F)
A. Matched α_1-antitrypsin data sets not included				
Log protein concentration	20.70	< 0.001	0.00	1.00
Log IL-8 concentration	25.29	< 0.001	0.12	0.74
Log myeloperoxidase concentration	28.16	< 0.001	3.45	0.08
IgG degradation	12.98	0.001	1.95	0.18
B. Matched α_1-antitrypsin data sets included				
Log protein concentration	19.19	< 0.001	0.02	0.89
Log IL-8 concentration	16.20	< 0.001	0.06	0.82
Log myeloperoxidase concentration	14.14	0.002	0.31	0.60
IgG degradation	8.21	0.012	0.28	0.62
Log α_1-antitrypsin	7.45	0.013	0.16	0.71
Log ratio of noncleaved to cleaved α_1-antitrypsin	31.74	< 0.001	0.09	0.77

Definition of abbreviation: IL = interleukin; Pr(> F) = tail area probability of the F value.

F tests are shown for the relationship of each biomarker to FEV_1. Part A: All markers were statistically significant when considered in isolation. Myeloperoxidase (log optical density) appeared to have the strongest relationship with FEV_1. Part B: When matched α_1-antitrypsin datasets were included in the analysis, all markers were again statistically significant when considered in isolation. The ratio of noncleaved to cleaved α_1-antitrypsin appeared to have the strongest marginal relationship with FEV_1; however, it did not improve prediction of FEV_1 beyond that obtained with myeloperoxidase expression and IgG degradation.

myeloperoxidase optical density. This is apparently in contradiction to the latter results, in which the strongest single relationship was between FEV_1 and the ratio of noncleaved to cleaved α_1-antitrypsin. This is an example of the backward elimination process failing to find the best overall solution for a given number of variables and is typical of the problems of interpreting multiple strongly correlated predictor variables. These results can best be interpreted as suggesting a strong relationship between both myeloperoxidase and the ratio of noncleaved to cleaved α_1-antitrypsin and FEV_1, but with little to choose between the markers.

DISCUSSION

Proteomic-based approaches have been used to discover biomarkers of disease in a wide range of diseases, including cancer (22), neurologic disorders (23), aging (24), heart disease (25), and lung disorders (26). In particular, 2-DE is a powerful proteomic tool with which to visualize modified forms of proteins and to compare proteomic profiles for diseased and nondiseased states (27). For CF, a number of proteomic studies have focused on analysis of protein expression in BALF (28–30). Altered expression of surfactant proteins SP-A and SP-D in BALF has been demonstrated by 2-DE and high-resolution mass spectrometry studies (10, 11, 31). For development of biomarker screening tests for respiratory diseases such as CF, particularly for point of care, sputum is a more amenable sample compared with BALF given it can be easily collected by noninvasive means. Sputum collected by saline induction has been shown to be a valuable tool for sampling and analysis of the contents of the lower airway (32, 33). In this study, saline-induced sputum profiles from adults with CF with an exacerbation and from children with CF with stable disease and preserved lung function were compared with profiles from adult and child control subjects. Furthermore, we have highlighted examples of protein isoforms that are differentially expressed between these different subject groups.

Cumulative lung damage in CF results from continual cycles of infection and inflammation that occur throughout an individual's lifetime (12), and is particularly characterized by a marked increase and persistent influx of neutrophils into the airways with consequent release of noxious mediators such as reactive oxygen species and proteolytic enzymes (34). At present, no quantitative test for point-of-care monitoring of CF lung disease is commercially available.

Despite the striking differences in 2-DE sputum protein profiles between the different subject groups, it could be speculated that such differences, particularly between adults with CF and children with CF, may be artifacts of sampling handling and processing times at different study sites. We believe this to be unlikely given the same protocols were standardized across all sites. Although there may have been slight variations in the time between sputum collection and the start of sample solubilization, albeit within 1 h, samples were always immediately placed on ice on collection. This would have minimized any further proteolytic degradation to an extent far less than what would have already occurred in the actual lung environment.

There is no "gold standard" for defining a pulmonary exacerbation. Diagnosis is based on a number of variables, including subjective measures of symptoms and clinical history (15, 16, 35). Measurement of FEV_1 is widely used to monitor changes in respiratory condition in CF (15, 16, 35). Although decreasing or increasing FEV_1 values help define a pulmonary exacerbation or recovery therefrom, respectively, we have shown by proteomic profiling of sputum that there are discrepancies between results when using proteomics and measurements of spirometry.

These studies demonstrate the feasibility of using biomarkers to monitor the dynamic biological processes in the lung that impact on tissue quality and ultimately respiratory function. Sputum profiles from adults with CF with an exacerbation demonstrated considerable protein expression differences after hospitalization and from control subjects. Here we have presented data for myeloperoxidase, α_1-antitrypsin, IgG degradation, and total protein concentration in comparison with IL-8, as biomarkers of lung exacerbation and examples of protein modifications that could be used in a prognostic/diagnostic test.

Although we have shown that increasing levels of myeloperoxidase, a protein involved in the inflammatory response by breaking down peroxide, are indicative of an exacerbation, decreasing levels are suggestive of improving pulmonary status. High levels of myeloperoxidase remained in certain adults with CF after hospitalization, suggesting insufficient clearance of inflammation. Adult CF subject 39, for example, presented a sputum profile at discharge indicating the possibility for further inflammation-induced tissue damage, despite a 23.6% increase in FEV_1 after hospitalization. This highlights the utility of biomarkers for helping to determine the length of drug treatment times and for regular monitoring for exacerbation and inflammation, particularly given current limitations in defining a pulmonary exacerbation (15, 16).

Comparative measures of IL-8, the major neutrophil chemoattractant peptide and a previously reported marker of inflammation in CF (33, 36, 37), show slightly different patterns relative to myeloperoxidase expression. For adults with CF, the differential expression at the exacerbation and discharge time points versus that of myeloperoxidase was greater for the majority of subjects with CF. The more notable discrepancies in expression of myeloperoxidase versus IL-8 were for subjects 12, 39, 44, and in particular subject 41, in whom minimal IL-8 was detected. One can only speculate as to reasons for these differences. It is unlikely that protein half-life accounts for these differences as

both proteins have estimated half-lives of 30 h (http://us.expasy.org/tools/protparam.html). It is possible that IL-8 and myeloperoxidase are biomarkers of slightly different stages of inflammation and/or exacerbation. IL-8 is more likely an indicator of early stage inflammation and exacerbation, given its role in recruitment of neutrophils, whereas myeloperoxidase possibly represents a biomarker of the underlying neutrophil influx as a consequence of inflammation at exacerbation. Further studies would be required to resolve this. In contrast to findings by Wolter and coworkers (38), but in agreement with others (33, 36, 37), our findings also suggest IL-8 as a biomarker of exacerbation (17). Nevertheless, our statistical analyses demonstrated that myeloperoxidase, and IgG degradation, were stronger predictors of FEV_1 than was IL-8.

A number of potential biomarkers of exacerbation in CF have been previously elucidated, particularly in association with inflammation and oxidative stress (32, 33, 39–48). Nevertheless, none of these markers have been developed into a rapid point-of-care test for patients with CF, often because of a paucity of statistical correlation (38). Myeloperoxidase has been reported as a marker for oxidative stress in inflammation (41, 49–53). With inflammation being a major cause of pulmonary deterioration (5, 34), and myeloperoxidase levels fluctuating as a measurable outcome of inflammation, myeloperoxidase represents an appealing biomarker of CF lung disease progression, which may be used in conjunction with FEV_1 measurements.

Cleavage of α_1-antitrypsin can be equally effective in monitoring pulmonary status in subjects with CF. α_1-Antitrypsin, a major protease inhibitor in the respiratory tract, is known to be cleaved by site-specific neutrophil-derived and bacterial proteases, including *P. aeruginosa* elastase (54). Expression of cleaved α_1-antitrypsin can distinguish between control subjects and subjects with CF and severity of disease (i.e., adults with CF with an exacerbation versus clinically stable children with CF). Many CF markers receive criticism given that patient-to-patient variability and day-to-day fluctuations in absolute biomarker expression can compromise their prognostic value. The relationship between subject status and age effects still held after analysis of the ratio of cleaved to noncleaved forms of α_1-antitrypsin, confirming that ratio measurements will help control for these variations. Like the other markers, including total protein concentration, we demonstrated a statistically significant correlation between α_1-antitrypsin and FEV_1, more so than IL-8; however, it was not as strong a predictor of FEV_1 as myeloperoxidase or IgG degradation.

Although there are a number of proteomic studies that have started to analyze BALF (10, 11, 28, 31), this is the first proteomic study to address changes in protein expression in sputum, particularly in the context of CF at different time points relative to healthy subjects. Studies of BALF in CF have demonstrated significant differences in protein patterns between healthy control and clinically stable subjects with CF, with the latter showing a predominance of low molecular weight proteins as we have observed in sputum (11). In agreement with our findings, studies of 2-D protein patterns of BALF, including analysis of proteolysis of SP-A, have also demonstrated how changes in protein patterns can be used to monitor molecular changes as a consequence of therapeutic intervention (10).

Longitudinal monitoring of IgG degradation could have important prognostic value for pulmonary status in chronically infected subjects with CF. Several human pathogens, including *P. aeruginosa*, *Haemophilus influenzae*, and various species of *Streptococcus*, encode proteases that cleave the heavy chains of IgG and IgA (55–58). These proteases are important bacterial virulence factors, allowing these pathogens to evade host defense mechanisms. Bacterially triggered degradation of immunoglobulins is thought to promote colonization and invasiveness at mucosal surfaces (55–58). Of the seven children with CF analyzed, IgG degradation was observed in sputum only from subjects 64 and 69, who both presented 2-DE profiles similar to those of adults with CF with an exacerbation. Degradation was observed in all the adults with CF with an exacerbation with degradation patterns closely relating to the proteomic patterns observed by 2-DE, with adult CF subjects 11, 41, and 44 clearly showing a reduced level of degradation at discharge. Degradation of IgA was also observed in sputum from all adults with CF with an exacerbation but not in any control subjects analyzed (data not shown). Although not extensively characterized in this study, the degradation of the immunoglobulin heavy chain is most likely due to the effective proteolytic activity of the bacterial proteases, concerning which a high degree of specificity for immunoglobulin molecules has been previously described (55, 56, 58).

Abnormalities in expression of IgG heavy chains have been associated with other inflammatory diseases such as chronic arthritis and rheumatoid arthritis, as well as γ heavy-chain diseases and amyloidosis (59–63). An impaired clearance of IgG_4 has also been reported with inflammatory bowel disease (62). Without knowledge of the cleavage sites, quantitation of these fragments in an immunodiagnostic test would be challenging. Nevertheless, as we have demonstrated, an electrophoresis-based assay could be developed as an alternative test for quantifying fragmentation patterns relating to lung disease, particularly given the strong correlation between IgG degradation and FEV_1.

Child CF subjects 64 and 69 had clinical symptoms and FEV_1 values within the normal range comparable to those of the other children with CF with stable disease and control subjects, yet their sputum protein profiles resembled those of adults with an exacerbation, with evidence of inflammation and protein degradation. Despite all clinical signs indicating a nonexacerbated state, without signs of acute inflammation, these two children with CF had higher levels of myeloperoxidase and IL-8, increased levels of cleaved α_1-antitrypsin, and IgG degradation, all of which indicated propensity for near-term exacerbation. Downstream diagnosis of flares of infection in only these two children with CF is interesting from a prognostic perspective; however, proof of causality, combined with a larger sample set and further longitudinal monitoring, is required.

Multiplexing these and/or other disease markers (33) for simultaneously monitoring infection, inflammation, and lung degradation might better predict exacerbation, thereby permitting early medical intervention before clinical symptoms develop, consequently minimizing tissue damage and lung pathology. Longitudinal studies, both in children and adults, will now be required to further address the prognostic utility of these markers. It is foreseeable that a prognostic test that incorporates these markers will also be of use for monitoring lung condition in other respiratory diseases, such as chronic obstructive pulmonary disorder, emphysema, chronic bronchitis, primary ciliary dyskinesia, and asthma, and may also assist as a useful or robust outcome measure in clinical trials.

Conflict of Interest Statement: A.J.S. is an employee of Proteome Systems Ltd. and has share options in this company. He is an inventor on provisional patents (pending) that have been filed around data presented in this manuscript. R.A.L. is an employee of Proteome Systems Ltd. and an inventor on provisional patents (pending) that have been filed around data presented in this manuscript. S.S.P. is an employee of Proteome Systems Ltd. and an inventor on provisional patents (pending) that have been filed around data presented in this manuscript. L.T.S. is an employee of Proteome Systems Ltd. and has share options in this company. S.K.P. is an employee of Proteome Systems Ltd. and an inventor on a provisional patent (pending) that has been filed around data presented in this manuscript. M.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.T.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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