Sputum Biomarkers of Inflammation in Cystic Fibrosis Lung Disease

Scott D. Sagel¹, James F. Chmiel², and Michael W. Konstan²

¹Department of Pediatrics, The Children's Hospital and University of Colorado at Denver and Health Sciences Center, Denver, Colorado; and ²Department of Pediatrics, Rainbow Babies and Children's Hospital and Case Western Reserve University School of Medicine, Cleveland, Ohio

Pulmonary biomarkers are being used more frequently to monitor disease activity and evaluate response to treatment in individuals with cystic fibrosis (CF). This article summarizes the current state of knowledge of biomarkers of inflammation relevant to CF lung disease, and the tools to measure inflammation, with specific emphasis on sputum. Sputum is a rich, noninvasive source of biomarkers of inflammation and infection. Sputum induction, through the inhalation of hypertonic saline, has expanded the possibilities for monitoring airway inflammation and infection, especially in individuals who do not routinely expectorate sputum. We critically examine the existing data supporting the validity of sputum biomarkers in CF, with an eye toward their application as surrogate endpoints or outcome measures in CF clinical trials. Further validation studies are needed regarding the variability of inflammatory biomarker measurements, and to evaluate how these biomarkers relate to disease severity, and to longitudinal changes in lung function and other clinical endpoints. We highlight the need to incorporate sputum collection, by induction if necessary, and measurement of sputum biomarkers into routine CF clinical care. In the future, pulmonary biomarkers will likely be useful in predicting disease progression, indicating the onset and resolution of a pulmonary exacerbation, and assessing response to current therapies or candidate therapeutics.

Keywords: cystic fibrosis; lung disease; biomarkers; inflammation; sputum

Cystic fibrosis (CF) lung disease is characterized by a self-perpetuating cycle of airway obstruction, chronic bacterial infection, and vigorous inflammation that results in bronchiectasis, progressive obstructive lung disease, and marked shortening of life expectancy (1, 2). Because airway inflammation plays a central role in the progression of CF lung disease, inflammatory biomarkers that can be used to monitor disease progression or evaluate response to therapy would be extremely valuable. With the increasing emphasis on development of candidate drugs that target airway inflammation in CF, we need to identify reliable biomarkers that indicate whether an antiinflammatory therapy is combating airway inflammation and, subsequently, yielding clinical benefit.

Although improvements in airway clearance and antibiotic therapies have been realized, the development of safe and effective antiinflammatory drugs has lagged behind (3). Corticosteroids and twice-daily high-dose ibuprofen were two of the first drugs studied in CF. Both have demonstrated clinical benefit,

but side effects and other considerations have limited their use (4–7). Results from previous clinical trials in CF indicate that antiinflammatory therapies may not result in immediate improvements in pulmonary function but could slow the rate of lung function decline and therefore extend survival (8). Investigations of antiinflammatory agents, in which the primary endpoint is rate of decline in lung function, would require that many patients be studied over a prolonged period of time (9). This poses a considerable challenge for translation of laboratory research advances into clinical treatments. Some means of screening candidate drugs more rapidly by use of robust biomarkers is urgently required. Biomarkers of inflammation could play a critical role in drug development. As measures of biologic activity, they could be used in early proof-of-concept studies to help screen potential drug candidates. Or, as correlates of clinical efficacy, they could lead to the selection of only the most promising agents for further study in phase III trials.

As discussed in the article by Mayer-Hamblett and colleagues in this symposium (pp. 370–377) (10), the ideal biomarker would be clinically and biologically relevant, reproducible, sensitive and specific to treatment effects, and feasible. At present, no single inflammatory biomarker clearly meets all of these criteria. Individual biomarkers will be appropriate in trials of targeted antiinflammatory therapeutic agents such as a specific anticytokine or antiprotease drugs. However, for clinical trials of antiinflammatory treatments that target multiple pathways of the inflammatory response or for therapeutic agents aimed at correcting CF transmembrane conductance regulator (CFTR) protein function or restoring airway surface liquid, a combination of biomarkers will likely prove more valuable. This article summarizes the current state of knowledge of airway inflammation in CF and the tools available to assess inflammation. We focus on sputum collection and examine the existing data that support the validity of sputum biomarkers. We conclude by suggesting additional validation studies to advance the utility and applicability of biomarkers of inflammation in the drug development process.

AIRWAY INFLAMMATION IN CF

For a more comprehensive description of airway inflammation in CF, the authors recommend a number of previous reviews of this subject matter (1, 2, 11, 12). The normal host inflammatory response consists of two phases: (1) activation by an inciting stimulus resulting in signal transduction, gene transcription, and altered cell function (proinflammatory mediator production), and (2) termination, which also includes signal transduction, gene transcription, and altered cell function (increased antiinflammatory and decreased proinflammatory mediator production, receptor endocytosis, production of soluble receptors, and return to baseline state). Abnormalities have been described for CF in both the activation and termination of the inflammatory response.

Although the lungs of newborns with CF are structurally normal, plugging of the bronchioles with secretions and hypertrophy of submucosal gland ducts appear by 4 months of age (13).

(Received in original form March 15, 2007; accepted in final form May 11, 2007)

Supported by NIH grants K23-RR018611-01 (S.D.S.), MO1-RR00069 (S.D.S.), U01-HL081335 (S.D.S.), and P30-DK27651 (J.F.C., M.W.K.), and the Cystic Fibrosis Foundation (S.D.S., J.F.C., M.W.K.).

Correspondence and requests for reprints should be addressed to Scott Sagel, M.D., The Children's Hospital, 1056 East 19th Avenue, Box B-395, Denver, CO 80218. E-mail: sagel.scott@tchden.org

Proc Am Thorac Soc Vol 4, pp 406–417, 2007

DOI: 10.1513/pats.200703-044BR

Internet address: www.atsjournals.org

Once challenged by viral or bacterial infection, high numbers of neutrophils are persistently recruited to the airway. Typically, normal host defense mechanisms are meant to eliminate an infection. This ultimately fails in the CF airway, and the subsequent inflammatory response becomes an independent and significant pathologic force (2). The CF airway contains large concentrations of inflammatory mediators and abundant amounts of neutrophils and neutrophil products, including oxidants and proteases. These overwhelm local defenses and contribute to lung injury. Lung secretions from patients with CF contain large concentrations of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-8 (14). All of these cytokines have the common characteristic that their synthesis is promoted by transcription factor nuclear factor (NF)- κ B, which is activated by cellular interaction with bacteria, bacterial products, and proinflammatory cytokines (15–17). Other pathways that may be involved in promoting the inflammatory response in CF include the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and activated protein (AP)-1 pathways (18–21).

Although the neutrophil is the predominant inflammatory cell present in the CF airway and any effective antiinflammatory therapy must address the neutrophil and its products directly or indirectly, it is not the only cell participating in the inflammatory response. The airway epithelial cell, which stands sentry between the host and its environment, clearly plays a major role in orchestrating the inflammatory response in the airway (22). The epithelial cell has important defense functions in the airway besides the obvious physical barrier that an intact epithelium provides and the basement membrane material that it secretes. Epithelial cells are the source of innate defense molecules, and they also direct the recruitment and activation of other cells of defense when appropriately stimulated. Some cytokine products of epithelial cells, such as TNF- α , IL-1 β , IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor, are produced in excess in CF, whereas some, such as IL-10 and RANTES (regulated on activation, normal T-cell expressed and secreted), appear to be reduced (14, 23–35).

Because airway epithelial cells normally express CFTR, absence of its normal function might have consequences that extend beyond ion transport in these cells. Defective cytokine production in airway epithelial cells, which are dependent on CFTR for normal secretory function, could explain why the disorders of the inflammatory response in CF seem limited to the respiratory tract (23, 29, 30, 36). Importantly, there is no increase in the frequency or severity of infection outside the respiratory tract, as would be found in patients with neutrophil defects or other primary immune deficiencies. Furthermore, patients with CF generally have normal immune responses to standard immunizations and often have elevated immunoglobulin concentrations.

In addition to airway epithelial cells, other inflammatory cells, such as macrophages, T lymphocytes, B lymphocytes, dendritic cells, and mast cells, are present in the CF airway in relative abundance and also are likely to contribute to the inflammatory response (37–41). Many of these cells express CFTR, but the pathophysiological link between dysfunctional CFTR and the enhanced inflammatory response in CF is not understood. There is supportive evidence suggesting a direct linkage between abnormal CFTR and the excessive airway inflammatory response. Inhibition of CFTR production by antisense oligonucleotides or inhibition of CFTR function by overexpression of the regulatory (R) domain of CFTR in epithelial cell lines results in increased activation of NF- κ B and increased secretion of IL-8 upon stimulation of these cells with proinflammatory stimuli (23, 24, 36, 42). Further studies using a pharmacologic inhibitor of CFTR

demonstrate that intact CFTR channel function is necessary for normal regulation of inflammatory cascades in epithelial cells (25). These results suggest that alterations in cellular physiology due to defective or deficient CFTR cause dysregulation of inflammatory pathways. Regardless of the relationship between CFTR and airway inflammation, it is clear that once set in motion, the inflammatory response is responsible for many of the secondary pathologic effects in CF and ultimately impacts survival. Until that time when a cure is realized, controlling the inflammatory response with drugs will be an important cornerstone of CF therapy. Because of the amplification that occurs in the inflammatory pathways, efficacy of an antiinflammatory agent targeted at any one step in the inflammatory cascade may result in attenuation of the overall inflammatory response.

Because of the excessive nature of the inflammatory response in the CF lung, many investigators have sought to determine abnormalities in either the activation or inhibition of the airway inflammatory response. Additional regulatory abnormalities may also occur that lead to excessive transcription of proinflammatory cytokine genes (18, 29, 30, 43, 44). Clinical and research observations that the inflammatory response occurs in the absence of detectable pathogens might suggest that the inflammatory response in the CF lung operates independently of an infectious stimulus. However, these findings may also be explained by failure to terminate the inflammatory response once the inciting stimulus has been eradicated and may actually represent an abnormal persistence of inflammation after clearance of early, transient infection (45, 46). Interestingly, some studies found that CF airways are relatively deficient in the counterregulatory molecules IL-10 and nitric oxide (NO) (29, 30, 47, 48), both of which preserve the function of inhibitory protein- κ B (I κ B), the inhibitor of NF- κ B. The decrease in NO production by CF airway epithelial cells appears to be secondary to a specific lesion in the signal transducer and activator of transcription (STAT)-1 pathway that leads to the activation of NO synthetase-2 (43, 44). In situations in which either IL-10 or NO or both might be deficient, less I κ B would be available to inhibit NF- κ B and proinflammatory mediator production would increase (18, 43, 44, 49, 50). Therefore, an imbalance between I κ B and NF- κ B in airway epithelia would result in the prolonged and excessive production of the mediators responsible for the damaging inflammation.

In addition, CF lung tissues appear to be deficient in peroxisome proliferator activating receptor (PPAR) (51). When activated, PPAR forms a heterodimer with activated retinoid X receptor (RXR), which is able to modulate inflammation in addition to other actions (52). PPAR exerts its attenuating effects typically by inhibiting NF- κ B activity via up-regulation of I κ B or by competition with NF- κ B for helicases (53). CF airway epithelial cell lines appear to have less PPAR γ activity than do non-CF airway epithelial cell lines (54). Thus, decreased PPAR expression also likely contributes to the imbalance between I κ B and NF- κ B that favors increased inflammation in CF. This has led some investigators to evaluate PPAR agonists in clinical trials in CF.

It seems apparent that the inflammatory lung damage in CF is much more complicated than an exuberant response to a persistent pathogen. Although bacterial infection plays an important role in stimulating the inflammatory response in CF, it also seems apparent that the inflammatory response itself is abnormal in several ways. These involve more than just overproduction of any one proinflammatory mediator. Several recent studies emphasize the concept that decreased antiinflammatory and counterregulatory activities are at least as important as increases in proinflammatory mediators in the overall pathophysiology of CF lung disease. The mechanisms proposed above are not mutually exclusive. It is likely that a combination of the

above processes fuel the aggressive and damaging inflammatory cascade. Therefore, a direct attack on inflammation itself seems warranted.

Given the life-limiting nature of CF, speeding drug development is imperative. Many trials are underway concurrently in search of therapies that will slow the progression of the lung disease. These studies often compete for a limited number of patients, and thus may result in the unintended consequence of slowing the development of potentially effective therapies. Biomarkers that more rapidly identify biologic activity and predict clinical response in CF would be very useful. Because the excessive inflammatory response in CF is primarily confined to the lung, investigators must necessarily study secretions and cells from the airway to better describe the association between defective CFTR and inflammation and the impact of antiinflammatory agents on the disease pathophysiology. Two different drugs may target completely separate areas of the inflammatory cascade, yet both may be effective in attenuating secondary disease processes. Clearly, some understanding of a drug's mechanism of action is necessary in selecting the most ideal outcome measures for a given clinical trial. It is unlikely that any one biomarker will be used as the sole surrogate outcome measure in a clinical study. Rather, it is more likely that a clinical trial will have to use a panel of biologic markers. The optimal array of biomarkers is likely to change from study to study depending on a drug's mechanism of action as described in later sections.

ASSESSING INFLAMMATION IN THE CF LUNG

Due to the localized nature of enhanced inflammation in CF, respiratory secretions from the lower airway are the optimal source of inflammatory cells and soluble mediators, and can be obtained directly or indirectly from the airways. The most common direct method is by performing bronchoscopy with bronchoalveolar lavage (BAL) or bronchial washing. Much of what has been learned about infection and inflammation in the CF lung has been through the use of BAL (14, 29, 45, 55–62) and much of the experience evaluating the “biologic efficacy” of therapeutics in CF has been through the use of BAL (46, 63–71). Thus, BAL and bronchial washing are considered by most investigators and clinicians to be the gold standard for assessing infection and inflammation in the CF lung. These procedures have been refined over time, with standard operating procedures used for multicenter studies. The reader is referred to a published standard operating procedure for performing BAL and for the processing of BAL fluid that has been adopted by the Cystic Fibrosis Foundation Therapeutics Development Network (CFF TDN) for their studies that use BAL (72). Additional details regarding the use of BAL, particularly in young children with CF, are described in the article by Davis and colleagues in this symposium (pp. 418–430) (73).

BAL offers the advantages of being able to be performed in any age group, and for its ability to sample the same area of the lung for studies that require repeat sampling (as is commonly done with intervention studies). This latter advantage results in the need for fewer subjects in clinical trials that use BAL. Disadvantages include the need for an experienced team to perform the procedure (to both obtain adequate samples and minimize risks to subjects), the need for sedation to perform the procedure (and its attendant risks), the inability to conveniently perform repeat sampling more than once or twice throughout an intervention study, and the high costs associated with performing the procedure. Moreover, correction for epithelial lining fluid dilution is often required for comparison purposes.

As a result of these drawbacks, investigators have become increasingly interested in less invasive or indirect methods for obtaining lower airway secretions. Expecterated sputum has been used for many years, but requires patients to have enough lung involvement and resultant respiratory secretions that allow regular sputum production. Thus, it is not very useful for examining inflammation in early lung disease, and in young children regardless of disease severity, since young children have difficulty expectorating sputum. To overcome the obstacle of obtaining sputum from subjects with milder lung disease, inducing a sputum specimen with hypertonic saline has been used to obtain lower airway secretions, and much effort has been expended in recent years in an attempt to validate the methodology of obtaining, processing, and assaying induced sputum. However, the use of induced sputum has its limitations as well, and other modalities for assessing lower airway inflammation need to be considered; these are briefly discussed in the remainder of this section.

Sputum induction is difficult to perform in patients aged 8 years and younger, and multicenter studies have largely limited inclusion to those aged 10 years or older. Moreover, repeated sampling of sputum obtained by induction (like expectorated sputum) may arise from different regions of the lung, and lead to increased variability of the measures of interest. This variability will likely increase the number of subjects needed for a study as compared with studies using BAL.

Other ways of indirectly assessing markers of inflammation in the lower airways have been considered, including exhaled gases and breath condensate, but they also have their limitations. Exhaled NO (eNO) is reduced in CF (47, 48); therefore, consideration could be given to measuring eNO in exhaled breath in a study of an antiinflammatory drug, such as the statins, that might impact the pathway leading to the up-regulation of NO. However, the use of this endpoint in other antiinflammatory trials has little utility. Although investigators have measured some inflammatory mediators in exhaled breath condensate (74–80), the usefulness of these measures as outcomes remains to be defined as well.

A systemic marker of lung inflammation would be ideal. Blood and urine can be obtained from subjects of any age and disease severity, and may reflect the status of inflammation throughout the lung, rather than one segment, as is assessed by BAL, or heterogeneous segments, as is assessed with sputum. However, a systemic marker may not be sensitive enough to detect a meaningful change in lung disease, given that the inflammatory response to infection in the CF lung is largely confined to the lung. Assessments in blood have included antibodies to *Pseudomonas aeruginosa* (81–83) or products of inflammation (84), neutrophil elastase α_1 -antiprotease complexes (63, 85), C-reactive protein (CRP) (85, 86), and various cytokines from serum or plasma (27, 87–97), and blood cells themselves (32, 33, 98–103). C-reactive protein is gaining increased recognition as a marker of inflammation for other diseases, but in CF, it is widely variable due to transient increases during pulmonary exacerbations (104, 105). Measures in urine have included elastin degradation products, such as desmosines (106), and products of arachidonic acid metabolism, such as leukotrienes (107). Further discussion of these systemic markers is beyond the scope of this article, but they are deserving of further exploration as markers of inflammation from the lung disease of CF.

The remainder of this article examines the existing data supporting the validity of sputum biomarkers of inflammation in CF, and what investigations are needed to further validate these markers as outcome measures in trials of antiinflammatory interventions in CF.

SPUTUM BIOMARKERS IN CF

For sputum biomarkers to be considered as accurate measures of biologic activity and outcome measures in CF clinical trials, they must fulfill certain key requirements of validation, as outlined by Mayer-Hamblett and colleagues (10), which are as follows: clinical and biologic relevance, reproducibility, sensitivity and specificity, and feasibility.

Clinical and Biologic Relevance

Biomarkers of inflammation and infection specifically reflect the chronic endobronchial infection and associated airway inflammation that are downstream consequences of CFTR protein dysfunction. Sputum has been used extensively to assess inflammation in the CF airway (34, 89, 91, 108–118). There are a number of inflammatory markers that either have been or could be measured in sputum (Table 1). Sputum is also a rich source of biomarkers of infection. Expecterated sputum provides an accurate measure of infection in the CF airway (119–121). Beyond serving as a means to ascertain lower airway microbial pathogens and quantitative bacterial counts by conventional culture, sputum can be used to identify bacterial species and bacterial proteins by molecular means (122–125).

An important step in determining the clinical and biologic relevance of sputum inflammatory biomarkers is to examine their relationship to other accepted clinical outcome measures. There is limited yet compelling evidence from small single-center studies supporting an association between sputum biomarkers and disease status in CF, defined either by pulmonary function measurements (109, 111, 115, 126–129), chest radiograph scores (112, 116), quality-of-life measures (114), or illness severity scores (e.g., Shwachman-Kulczycki score) (89). Sagel and coworkers have shown significant correlations between FEV₁ and a number of induced sputum inflammatory measures, including neutrophil counts, IL-8, neutrophil elastase, and matrix metalloproteinase (MMP)-9 in clinically stable children with CF with normal to mildly abnormal lung function (126, 128). Similarly, Kim and colleagues demonstrated significant correlations between pulmonary function and both IL-8 and myeloperoxidase

in older subjects with more advanced CF lung disease (129). Furthermore, there are recent data that the correlations between sputum markers of inflammation and pulmonary function are statistically significant across a diverse CF population. A retrospective review of multiple clinical studies conducted through the CFF TDN afforded the unique opportunity to investigate associations between sputum biomarkers and pulmonary function in over 250 subjects across multiple study centers (130). This study reported significant negative correlations between FEV₁ and spontaneously expectorated sputum inflammatory markers, including free elastase, IL-8, neutrophil counts, and percentage of neutrophils. Free elastase and neutrophil counts were able to explain the greatest amount of variation in FEV₁ across patients.

Further evidence that sputum biomarkers are clinically relevant include investigations that demonstrate elevated sputum concentrations of neutrophils (131) and inflammatory mediators (34, 132, 133) in patients experiencing an acute pulmonary exacerbation compared with those who are clinically stable. Of note, other groups have not found significant differences in sputum inflammatory markers between periods of exacerbation and stability (114, 131, 134). Elevated sputum inflammatory markers have also been demonstrated in those with chronic *P. aeruginosa* endobronchial infection (115, 132).

Although cross-sectional analyses are most commonly reported in the literature, there are emerging longitudinal natural history data correlating sputum biomarker changes with disease progression in CF. Longitudinal analyses conducted by Mayer-Hamblett and coworkers revealed significant associations between increases in free elastase and decreases in FEV₁ among the subset of subjects with CF who were randomized to placebo and underwent more than one measurement in these clinical trials (130). In addition, changes in sputum DNA concentrations at two discrete time points were correlated with changes in pulmonary function in one of the previously mentioned single-center studies (129). These and further longitudinal analyses are essential for the validation of these sputum biomarkers as correlates of disease severity.

TABLE 1. CANDIDATE BIOMARKERS OF CYSTIC FIBROSIS AIRWAY INFLAMMATION

Inflammatory cells	Neutrophils, macrophages, lymphocytes, eosinophils, mast cells, dendritic cells, airway epithelial cells
Cytokines	IL-1 β , IL-6, IL-8, IL-10, IL-17, IL-23, TNF- α , transforming growth factor β ₁ , IFN- γ , GM-CSF, G-CSF, growth-related oncogene α , RANTES
Cytokine antagonists	IL-1 receptor antagonist, soluble TNF receptor II
Neutrophil chemoattractants	IL-8, leukotriene (LT)B ₄ , complement-derived attractants (c5a)
Proteases	Elastase, matrix metalloproteinases (MMP-9), cathepsin G, proteinase 3
Antiproteases	α ₁ -Antitrypsin, tissue inhibitors of metalloproteinases (TIMP-1), SLPI, elafin, α ₂ -macroglobulin
Markers of oxidative stress	Protein carbonyls, 3-nitrotyrosine, 3-chlorotyrosine, 8-isoprostane
Antioxidants	Superoxide dismutase, glutathione, catalase, tocopherols (vitamin E), β -carotene
Other neutrophil products	DNA, myeloperoxidase, human neutrophil lipocalin
Plasma exudate markers	Albumin, fibrinogen
Antimicrobial proteins	Lysozyme, lactoferrin, secretory component of IgA, bactericidal permeability-increasing protein, human cationic antimicrobial protein 18
Antimicrobial peptides	Defensins, cathelicidin LL37
Nitric oxide metabolites and pathway	Nitrites, nitrates, nitrotyrosine, arginase, ammonium (NH ₄ ⁺)
Eicosanoids	LTB ₄ , LTC ₄ , LTD ₄ , LTE ₄ , prostaglandin (PG)D ₂ , PGE ₂ , 8-isoprostane, thromboxane B ₂
Mucins	MUC5AC, MUC5B
Adhesion molecules	Soluble intracellular adhesion molecule
Markers of structural airway injury	Elastin and collagen degradation products
Components of signaling cascades	Nuclear factor- κ B, ERK, MAPK, JAK/STAT, PPAR

Definition of abbreviations: ERK = extracellular signal-regulated kinase; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; JAK = Janus kinase; MAPK = mitogen-activated protein kinase; PPAR = peroxisome proliferator activating receptor; RANTES = regulated on activation, normal T-cell expressed and secreted; SLPI = secretory leukoprotease inhibitor; STAT = signal transducer and activator of transcription; TNF = tumor necrosis factor.

In summary, sputum biomarkers of inflammation and infection are clinically and biologically relevant in CF. Therapies that either modify the progression of CF lung disease or treat the consequences (i.e., infection and inflammation) will likely directly or indirectly attenuate biomarkers of lung inflammation and infection. The responsiveness of these biomarkers will be highly dependent on the stage of underlying lung disease. For instance, we know that it is extremely difficult to eradicate mucoid forms of *P. aeruginosa* from the lower airways of those who are chronically infected with this organism (135). Similarly, it may be very difficult to reduce the inflammatory burden in those with severe bronchiectasis and end-stage lung disease. Significant changes in inflammatory biomarkers are more likely to occur before lung disease becomes irreversible. Ideally, the concentrations and/or activity of inflammatory mediators would decrease in response to a drug with direct or indirect antiinflammatory effects. However, even maintaining inflammation at its present levels could yield clinical benefit (see the discussion in SENSITIVITY AND SPECIFICITY TO TREATMENT EFFECTS). Based on previous trials, we expect that biomarkers of inflammation would change over months (i.e., 1–3 mo) rather than days or even weeks (136, 137).

Reproducibility

There is limited information about the variability and repeatability of cellular and inflammatory markers in sputum of persons with CF. Data from two published studies demonstrate reasonably good reproducibility of induced sputum cell counts and neutrophil percentages in clinically stable children (138) and adults (139) with CF. In addition, measurements of inflammatory mediators, including cytokines and proteases in CF sputum, are fairly reproducible (139, 140). Importantly, these data demonstrate that within-patient variability is small in comparison to the between-patient variability, a necessary characteristic ensuring that estimates of between-patient differences are not missed or diluted (140). Only one group has examined the reproducibility of sputum bacterial cultures in CF (140). We need more precise information on reproducibility of sputum biomarkers of inflammation and/or infection before they can be routinely used as an outcome measure in CF clinical trials.

Although measurements of biomarkers of inflammation in sputum samples obtained from the same patient on different occasions are reproducible, these measurements can still vary considerably, especially among different persons with CF. There are a number of reasons for these intersubject differences, including the following: (1) heterogeneity of airway inflammation across lung regions (141), (2) inherent biologic variability due to changes in clinical status, (3) sputum collection (i.e., spontaneous expectoration vs. induction) and processing methodologies used, and (4) laboratory assay techniques (e.g., enzyme-linked immunosorbent assay) used. For instance, the method to disperse and homogenize the sputum can have important effects on the detection and quantitation of inflammatory mediators in sputum supernatants. There is a fairly extensive body of literature examining the effects of various sputum-processing techniques, in particular the use of dithiothreitol (DTT) as a solubilizing agent, on sputum inflammatory mediators (142–148). Although these and other studies (149–152) indicate that DTT is satisfactory for liquefying sputum and dispersing cells in persons with CF, it is important to recognize that DTT can also interfere with the detection of several inflammatory mediators (147, 148).

This highlights the need to use consistent, standardized approaches in regard to both sputum collection and sputum processing. To this end, the CFF TDN has developed standard operating procedures both for sputum induction and sputum processing. These standard operating procedures are available

on request at the CFF TDN (e-mail: tdncc@seattlechildrens.org), and provide guidelines for a uniform method of collecting sputum by induction and for processing expectorated or induced sputum. The CFF TDN has established centralized core laboratories for sputum cytology (Case Western Reserve University) and inflammatory mediator analysis (The Children's Hospital/University of Colorado at Denver and Health Sciences Center). Furthermore, the TDN has developed training videos and has sponsored on-site training visits and ongoing quality-control evaluations for certifying research personnel in performing these procedures. All of these efforts are intended to improve reproducibility and decrease variance, and help to ensure that sputum inflammatory biomarkers are reliable and useful.

Sensitivity and Specificity to Treatment Effects

A key step in the validation of sputum biomarkers is to demonstrate that they are sensitive toward measuring changes in the mechanistic pathway they are intended to measure. Assessing sensitivity for sputum biomarkers is best done in the context of a successful therapeutic intervention that can significantly alter the infection and inflammatory process. The best study that provides proof of concept demonstrating changes in sputum biomarkers after a proven therapeutic intervention was a multicenter study of sputum induction before and after intravenous antibiotic treatment for acute pulmonary exacerbations in CF (118). In this study, mean FEV₁ improved by 0.3 L after antibiotic treatment, which was both clinically meaningful and statistically significant. This clinical improvement was associated with a 2- to 4-log reduction in lower airway bacterial density, and an approximate 0.5-log reduction in several induced sputum inflammatory markers, including neutrophil counts, IL-8, and neutrophil elastase. The observed correlations between changes in pulmonary function and induced sputum markers of lower airway infection and inflammation provide evidence that these markers are sensitive to the treatment effects of intravenous antibiotics and augmented mucus clearance. We recognize that intravenous antibiotic treatment of a pulmonary exacerbation is a very robust therapy for demonstrating acute changes in these markers.

Beyond this particular study, there are limited data demonstrating meaningful changes in sputum biomarkers of inflammation after other proven therapeutic interventions in CF. Three studies have examined the effects of recombinant human DNase (rhDNase) (Pulmozyme; Genentech, San Francisco, CA) on airway inflammation, assessed in sputum (136, 153, 154). Shah and colleagues found modest reductions in sputum neutrophil elastase activity (136), whereas the other two reported no significant changes in neutrophil counts (153) or inflammatory mediators (154) over 1 to 3 months of Pulmozyme treatment. However, over a 3-year period, rhDNase prevented an increase in several markers of lower airway inflammation, as measured in BAL, which was observed in untreated patients (70). In other words, a therapy could have a positive impact on airway inflammation either by reducing levels of proinflammatory mediators in the short term (weeks to months) or by preventing an increase in inflammation that might occur over years. Similarly, azithromycin might exert an antiinflammatory effect by preventing a worsening of inflammation over time. For instance, in the U.S. azithromycin trial, there were modest differences in sputum elastase between the placebo and treated groups at the end of treatment in favor of the azithromycin group (Figure 1) (137). This difference was mainly attributed to an increase in sputum elastase over 6 months in the placebo group (0.2 log₁₀ µg/ml), whereas levels remained stable in the azithromycin group.

Sputum biomarkers of inflammation have been included as outcome measures in several other recent clinical trials

Figure 1. Sputum neutrophil elastase activity measurements from the U.S. multicenter azithromycin clinical trial. Mean elastase levels in the azithromycin and placebo groups were similar at baseline. There was a significant difference in elastase levels between the two groups at the end of treatment, mainly due to an increase in sputum elastase in the placebo group. Adapted by permission from Reference 137.

(155–162). The results have been mixed, with some therapeutic agents reducing sputum inflammatory mediators and others showing no discernable antiinflammatory effect. Although these studies are adding to our knowledge of the role of inflammatory biomarkers in CF clinical trials, the sensitivity of any particular biomarker will mainly be established in the setting of a drug that is clearly successful at modifying the underlying inflammatory process.

To date, there is a paucity of information demonstrating the specificity of sputum biomarkers of inflammation. At a qualitative level, specificity means that sputum biomarkers will not change or worsen in response to an unsuccessful treatment. Full validation of sputum biomarkers will require use of placebo or control groups to demonstrate that the biomarkers can distinguish changes in response to drug from natural changes due to the disease course. Data should be emerging from ongoing or forthcoming antiinflammatory trials. For instance, the CFF TDN recently conducted a multicenter study examining the effects of ibuprofen on inflammatory mediators in induced sputum from subjects with mild to moderate CF lung disease. One of the mechanisms of action of high-dose ibuprofen is reducing neutrophil migration (163). If 1 month of high-dose ibuprofen leads to a reduction in induced sputum neutrophil counts, this will raise our confidence in the sensitivity of sputum biomarkers. In contrast, if an antiinflammatory agent proves unsuccessful or even detrimental, sputum biomarkers that are specific will remain largely unaffected or will change in the wrong direction. In summary, there is a clear need to more fully describe the sensitivity and specificity of sputum biomarkers to treatment in multiple therapeutic areas over different time courses.

Feasibility

Sputum collection is a simple, noninvasive way to qualitatively and quantitatively assess lower airway inflammation and infection with minimal associated patient burden. Spontaneous sputum expectoration is generally limited to adolescents and adults with CF or to those with more advanced degrees of lung disease. Sputum induction, through the inhalation of hypertonic saline, has expanded the possibilities for monitoring airway inflammation and infection, especially in those individuals who do not routinely expectorate sputum. Sputum induction is mainly feasible and generally successful in children with CF who are 10 years and older. Because the cellular and biochemical composition of induced sputum does not differ significantly from spontaneously

produced sputum (117, 164), repeated sputum collections, either by spontaneous expectoration or induction, provide the ability to monitor the impact of an intervention on airway inflammation and infection. One advantage of induced sputum in comparison to spontaneously expectorated sputum is that larger sample volumes obtained by induction allow for more extensive measurements of inflammatory markers. On the other hand, time and costs obviously favor spontaneous expectoration. The total sputum induction time generally lasts 30 to 45 minutes because of the need for pretreatment with albuterol, set-up time, and safety monitoring (FEV₁ or peak flows) throughout the procedure. Trained personnel are required, because respiratory therapists generally perform the procedure. Also, consecutive sputum inductions within a short time interval can cause an increase in the percentage of neutrophils in sputum (165, 166). It is recommended that repeat inductions be separated by at least 48 hours (167). Therefore, sputum induction is more relevant for early-phase clinical trials or for studies involving subjects with milder lung disease.

The main factor limiting the routine use of sputum collection in multicenter CF clinical trials is the need for specialized laboratories and trained technologists to process the samples. It generally takes between 1 and 2 hours to thoroughly homogenize and aliquot sputum samples. Importantly, examination of sputum cell counts is limited by the need to process samples within 2 hours of collection (168). However, freezing and delayed processing appear to be options for analysis of biochemical mediators. Recent data suggest that there are no significant effects of freezing and thawing on the cytokines IL-8 and TNF- α (169). Similarly, the TDN inflammatory mediator core laboratory has found that neutrophil elastase activity is robust and not significantly hampered by delayed processing or freezing and thawing of samples. These findings are particularly relevant to multicenter trials, by assuring that sputum samples can be collected, frozen, and shipped to centralized sputum processing laboratories.

Finally, for sputum biomarkers to become more practical and clinically useful, the entire sputum induction procedure will need to be shortened and sputum processing will need to be technically simplified (e.g., using rapid automated methods), permitting sputum collection to be integrated into routine clinical care, similar to pulmonary function testing and chest imaging. At the present time, most CF centers only collect samples from those subjects who are able to spontaneously expectorate sputum, whereas sputum induction is mainly reserved for research purposes. With improved procedures, we hope that more CF care centers will be able to incorporate this step into clinical care in the near future to obtain more extensive longitudinal microbiologic and inflammatory data on their patients. In this setting, sputum biomarkers could be used for a variety of purposes in CF (Table 2).

TABLE 2. POTENTIAL UTILITY OF SPUTUM BIOMARKERS IN CYSTIC FIBROSIS

Diagnostic test (particularly of airway infection)
Monitor disease activity
Provide mechanism of action of drug candidates
Predict rapid disease progression
Indicate the onset and resolution of an acute pulmonary exacerbation
Prescribe targeted and individualized therapies
Evaluate the response to treatment
Means of patient stratification for CF clinical trials
Serve as candidate surrogate endpoints in early-phase CF clinical trials

Definition of abbreviation: CF = cystic fibrosis.

NEXT STEPS

We have presented supportive evidence that sputum biomarkers of inflammation and infection are clinically and biologically relevant, repeatable, sensitive to treatment effects, and generally feasible. Yet, many questions remain unanswered. At present, we do not have sufficient data to conclude which sputum biomarkers are the best and most informative to measure in individuals with CF. For instance, is it preferable to determine absolute neutrophil load or levels of inflammatory mediators (i.e., IL-8 or elastase), which may be more representative of the activation state of these cells? What is the best way to measure the activation state of neutrophils in the CF airway? And once we determine which biomarkers are most instructive, it will be necessary to quantify the level of change in the biomarkers needed to produce a change in clinical status. In other words, what constitutes a "minimal clinically meaningful difference" in sputum inflammatory measurements? Would even a 20% reduction in sputum neutrophils or associated inflammatory mediators correlate with either an improvement in respiratory status or a delay in disease progression?

To begin to answer these questions, further studies are required. More research is needed on both the short- and long-term variability of sputum inflammatory markers and sputum bacterial density among a larger number of carefully phenotyped subjects with CF of varying ages. Preferably, these studies would be conducted at multiple CF centers rather than at a single site. These data would permit a broader application of sputum biomarkers. We need to more rigorously examine how factors such as age, sex, CF genotype, modifier genes, and host microbial pathogens influence the relationships between sputum biomarkers, lung function, and lung structure (e.g., ascertained on high-resolution computed tomography scans). Also, future studies should investigate longitudinal associations between changes in sputum biomarkers and other clinical endpoints besides FEV₁, such as quality of life or patient-reported symptom scores. Furthermore, we are in need of reliable markers of airway structural change, such as elastin or collagen breakdown products, because these may provide the best evidence of evolving or worsening bronchiectasis in persons with CF.

Finally, all previous studies have correlated individual biomarkers with clinical status. Because so many proteins and inflammatory pathways are involved in CF, it seems unlikely that

one single biomarker will successfully foretell clinical outcomes. A validated panel of biomarkers is almost certainly required. In fact, investigators from other disciplines are finding that panels of biomarkers, rather than individual ones, are providing improved diagnostic power to predict cardiovascular disease risk (170) and prognosticate a patient's risk of myocardial infarctions and death (171). A CF lung biomarker panel could be a more sensitive composite endpoint toward demonstrating biologic efficacy as compared with individual biomarkers. A CF biomarker panel would be critical in the drug development process for tracking rapid disease progression and capturing response to treatment. The success of this panel in predicting clinical response could ultimately position biomarkers of inflammation as potential surrogate endpoints in later-stage clinical trials, or at a minimum, provide the necessary data to proceed with confidence from early-phase proof-of-concept studies to confirmatory trials. Based on the available literature, we anticipate that a CF lung injury biomarker panel might include a combination of three to five of any of the following key biomarkers: neutrophils; cytokines (IL-1 β , IL-8, TNF- α); proteases (neutrophil elastase, MMP-9); antiproteases (α_1 -antitrypsin, TIMP-1 [tissue inhibitor of metalloproteinase-1], SLPI [secretory leukoprotease inhibitor]); and other neutrophil markers (DNA, myeloperoxidase). And rather than simply reporting the values of biomarkers, it will be much more clinically meaningful to attempt to define specific cutoff ranges for any proposed biomarkers.

Our working group has outlined several key recommendations and research questions that remain to be addressed (Table 3).

Relationship between Airway Infection and Inflammation in CF

As we attempt to further validate biomarkers of airway inflammation, we believe it will be important to monitor infection as well, especially in subsequent clinical trials. Figure 2 addresses the critical relationship between airway inflammation and infection and examines how corresponding changes in markers of inflammation and infection may affect persons with CF. The ideal treatment reduces both inflammation and infection (quadrant I). A treatment that leads to an increase in measures of inflammation and infection would obviously be detrimental (quadrant III). A treatment that reduces inflammation at the cost of increasing infection is probably unfavorable (quadrant II). Yet, the converse scenario, where infection is reduced and inflammation

TABLE 3. KEY RECOMMENDATIONS OF THE INFLAMMATORY BIOMARKERS WORKING GROUP

Procedural Issue	Outstanding Questions
Sputum collection	The influence of different nebulizer systems (high-output ultrasonic, low-output ultrasonic, jet) on quantity of sputum induced, sputum cell counts, and biochemical mediators
Sputum processing	The effect of dithiothreitol (vs. saline or enzyme mixtures) on many inflammatory mediators The effect of delay in processing (i.e., immediate freezing) on many inflammatory mediators
Validation of sputum biomarkers	Short-term reproducibility (weeks) of measurements of many inflammatory mediators Longer term variability (months) of measurements of many inflammatory mediators Further evaluate how biomarkers relate to disease severity, and to longitudinal changes in lung function and lung structure Need to establish the minimal clinically meaningful difference in sputum neutrophil counts or inflammatory mediators Identify reliable markers of airway structural change such as elastin or collagen breakdown products Develop a CF lung injury biomarker panel consisting of three to five proteins with proposed cutoff ranges
Other biomarker sources	The value of systemic (plasma, serum, urine) markers of inflammation to evaluate response to treatment or predict disease progression The value of exhaled gases (nitric oxide) or breath condensate to evaluate response to treatment or predict disease progression

Definition of abbreviation: CF = cystic fibrosis.

Figure 2. Schematic of possible effects of drug candidates on sputum markers of inflammation and infection and associated clinical consequences.

increases, might have positive or negative consequences (quadrant IV).

CONCLUSIONS

Until the time when therapies aimed at repairing or restoring the basic gene or protein defect are realized, limiting the effects of the inflammatory process will remain important in slowing the decline in lung function and thus prolonging survival in individuals with CF. Biomarkers of inflammation will almost certainly demonstrate a beneficial effect of any successful anti-inflammatory treatment. This is an active field of research and further studies addressing many of the issues raised in this article are already underway.

Conflict of Interest Statement: S.D.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.F.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.W.K. received/receives honoraria for serving as a consultant and/or for membership on advisory boards for Aradigm, Boehringer Ingelheim, Chiron, Debiopharm, Genentech, Novartis, PTC Therapeutics, and ZLB-Behring.

Acknowledgment: The authors thank Jay Hilliard (Case Western Reserve University) and John Beamer (TDN Coordinating Center) who gave very elegant presentations regarding sputum processing and importance of quality assurance at the Inflammatory Biomarkers Working Group meeting. They also want to recognize the following meeting participants: Chris Oermann and Marcia Katz (Baylor College of Medicine), Pamela Zeitlin (Johns Hopkins University), Hank Dorkin (Massachusetts General Hospital), Carol Conrad (Stanford University), Randy Young (University of Alabama), Doug Conrad (University of California, San Diego), Cori Daines (University of Cincinnati), Peggy Emmett (University of Colorado), Cynthia Williams (University of Minnesota), George Retsch-Bogart (University of North Carolina), Elizabeth Hartigan (University of Pittsburgh), Ted Liou (University of Utah), Ron Gibson (University of Washington), Tom Ferkol (Washington University), Nicole Mayer-Hamblett (TDN Coordinating Center), and Preston Campbell and Heba Barazi (Cystic Fibrosis Foundation Therapeutics, Inc.). Finally, the authors gratefully acknowledge the critical review of this manuscript by Bonnie Ramsey, M.D., Lynn Rose, Ph.D., Nicole Mayer-Hamblett, Ph.D., and Frank Accurso, M.D.

References

- Davis PB, Drumm M, Konstan MW. Cystic fibrosis. *Am J Respir Crit Care Med* 1996;154:1229–1256.
- Chmiel JF, Berger M, Konstan MW. The role of inflammation in the pathophysiology of CF lung disease. *Clin Rev Allergy Immunol* 2002; 23:5–27.
- Chmiel JF, Konstan MW. Anti-inflammatory medications for cystic fibrosis lung disease: selecting the most appropriate agent. *Treat Respir Med* 2005;4:255–273.
- Eigen H, Rosenstein BJ, FitzSimmons S, Schidlow DV. A multicenter study of alternate-day prednisone therapy in patients with cystic fibrosis. Cystic Fibrosis Foundation Prednisone Trial Group. *J Pediatr* 1995;126:515–523.
- Konstan MW, Byard PJ, Hoppel CL, Davis PB. Effect of high-dose ibuprofen in patients with cystic fibrosis. *N Engl J Med* 1995;332:848–854.
- Oermann CM, Sockrider MM, Konstan MW. The use of anti-inflammatory medications in cystic fibrosis: trends and physician attitudes. *Chest* 1999;115:1053–1058.
- Lai HC, FitzSimmons SC, Allen DB, Kosorok MR, Rosenstein BJ, Campbell PW, Farrell PM. Risk of persistent growth impairment after alternate-day prednisone treatment in children with cystic fibrosis. *N Engl J Med* 2000;342:851–859.
- Konstan MW, Davis PB. Pharmacological approaches for the discovery and development of new anti-inflammatory agents for the treatment of cystic fibrosis. *Adv Drug Deliv Rev* 2002;54:1409–1423.
- Davis PB, Byard PJ, Konstan MW. Identifying treatments that halt progression of pulmonary disease in cystic fibrosis. *Pediatr Res* 1997; 41:161–165.
- Mayer-Hamblett N, Ramsey BW, Kronmal RA. Advancing outcome measures for the new era of drug development in cystic fibrosis. *Proc Am Thorac Soc* 2007;4:370–377.
- Sagel SD, Accurso FJ. Monitoring inflammation in CF: cytokines. *Clin Rev Allergy Immunol* 2002;23:41–57.
- Courtney JM, Ennis M, Elborn JS. Cytokines and inflammatory mediators in cystic fibrosis. *J Cyst Fibros* 2004;3:223–231.
- Bedrossian CW, Greenberg SD, Singer DB, Hansen JJ, Rosenberg HS. The lung in cystic fibrosis. a quantitative study including prevalence of pathologic findings among different age groups. *Hum Pathol* 1976; 7:195–204.
- Bonfield TL, Panuska JR, Konstan MW, Hilliard KA, Hilliard JB, Ghnaim H, Berger M. Inflammatory cytokines in cystic fibrosis lungs. *Am J Respir Crit Care Med* 1995;152:2111–2118.
- DiMango E, Ratner AJ, Bryan R, Tabibi S, Prince A. Activation of NF-kappaB by adherent *Pseudomonas aeruginosa* in normal and cystic fibrosis respiratory epithelial cells. *J Clin Invest* 1998;101:2598–2605.
- Li J, Johnson XD, Iazovskaia S, Tan A, Lin A, Hershenson MB. Signaling intermediates required for NF-kappa B activation and IL-8 expression in CF bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L307–L315.
- Barnes PJ. Transcription factors in airway diseases. *Lab Invest* 2006;86: 867–872.
- Kraynack NC, Corey DA, Elmer HL, Kelley TJ. Mechanisms of NOS2 regulation by Rho GTPase signaling in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L604–L611.
- Bozinovski S, Jones JE, Vlahos R, Hamilton JA, Anderson GP. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFkappa B and AP-1 in vivo. *J Biol Chem* 2002;277: 42808–42814.
- Delgado MA, Poschet JF, Deretic V. Nonclassical pathway of *Pseudomonas aeruginosa* DNA-induced interleukin-8 secretion in cystic fibrosis airway epithelial cells. *Infect Immun* 2006;74:2975–2984.
- Sadikot RT, Christman JW, Blackwell TS. Molecular targets for modulating lung inflammation and injury. *Curr Drug Targets* 2004;5:581–588.
- Chmiel JF, Davis PB. Inflammatory responses in the cystic fibrosis lung. In: Kirk KL, Dawson DC, editors. The CFTR chloride channel, 1st ed. Georgetown, TX: Landes Bioscience; 2003. pp. 160–180.
- Kube D, Sontich U, Fletcher D, Davis PB. Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L493–L502.
- Saba S, Soong G, Greenberg S, Prince A. Bacterial stimulation of epithelial G-CSF and GM-CSF expression promotes PMN survival in CF airways. *Am J Respir Cell Mol Biol* 2002;27:561–567.
- Perez A, Issler AC, Cotton CU, Kelley TJ, Verkman AS, Davis PB. CFTR inhibition mimics the cystic fibrosis inflammatory profile. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L383–L395.
- Ruef C, Jefferson DM, Schlegel-Haueter SE, Suter S. Regulation of cytokine secretion by cystic fibrosis airway epithelial cells. *Eur Respir J* 1993;6:1429–1436.
- Jensen PO, Moser C, Kharazmi A, Presler T, Koch C, Hoiby N. Increased serum concentration of G-CSF in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* pneumonia. *J Cyst Fibros* 2006;5: 145–151.
- Bedard M, McClure CD, Schiller NL, Francoeur C, Cantin A, Denis M. Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. *Am J Respir Cell Mol Biol* 1993;9:455–462.

29. Bonfield TL, Konstan MW, Burfeind P, Panuska JR, Hilliard JB, Berger M. Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am J Respir Cell Mol Biol* 1995;13:257-261.
30. Bonfield TL, Konstan MW, Berger M. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *J Allergy Clin Immunol* 1999;104:72-78.
31. Soltyz J, Bonfield T, Chmiel J, Berger M. Functional IL-10 deficiency in the lung of cystic fibrosis (Cftr(-/-)) and IL-10 knockout mice causes increased expression and function of B7 costimulatory molecules on alveolar macrophages. *J Immunol* 2002;168:1903-1910.
32. Moss RB, Hsu YP, Olds L. Cytokine dysregulation in activated cystic fibrosis (cf) peripheral lymphocytes. *Clin Exp Immunol* 2000;120:518-525.
33. Moss RB, Bocian RC, Hsu YP, Dong YJ, Kemna M, Wei T, Gardner P. Reduced IL-10 secretion by CD4+ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR). *Clin Exp Immunol* 1996;106:374-388.
34. Osika E, Cavaillon JM, Chadelat K, Boule M, Fitting C, Tournier G, Clement A. Distinct sputum cytokine profiles in cystic fibrosis and other chronic inflammatory airway disease. *Eur Respir J* 1999;14:339-346.
35. Schwiebert LM, Estell K, Propst SM. Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *Am J Physiol* 1999;276:C700-C710.
36. Kube DM, Fletcher D, Davis PB. Relation of exaggerated cytokine responses of CF airway epithelial cells to PAOI adherence. *Respir Res* 2005;6:69.
37. McDonald TV, Nghiem PT, Gardner P, Martens CL. Human lymphocytes transcribe the cystic fibrosis transmembrane conductance regulator gene and exhibit CF-defective CAMP-regulated chloride current. *J Biol Chem* 1992;267:3242-3248.
38. Yoshimura K, Nakamura H, Trapnell BC, Chu CS, Dalemans W, Pavirani A, Lecocq JP, Crystal RG. Expression of the cystic fibrosis transmembrane conductance regulator gene in cells of non-epithelial origin. *Nucleic Acids Res* 1991;19:5417-5423.
39. Krauss RD, Berta G, Rado TA, Bubien JK. Antisense oligonucleotides to CFTR confer a cystic fibrosis phenotype on B lymphocytes. *Am J Physiol* 1992;263:C1147-C1151.
40. Hubeau C, Lorenzato M, Couetil JP, Hubert D, Dusser D, Puchelle E, Gaillard D. Quantitative analysis of inflammatory cells infiltrating the cystic fibrosis airway mucosa. *Clin Exp Immunol* 2001;124:69-76.
41. McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, Steele C, Finder JD, Pilewski JM, Carreno BM, Goldman SJ, et al. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *J Immunol* 2005;175:404-412.
42. Perez A, Risma KA, Eckman EA, Davis PB. Overexpression of R domain eliminates CAMP-stimulated Cl- secretion in 9/HTEo- cells in culture. *Am J Physiol* 1996;271:L85-L92.
43. Steagall WK, Elmer HL, Brady KG, Kelley TJ. Cystic fibrosis transmembrane conductance regulator-dependent regulation of epithelial inducible nitric oxide synthase expression. *Am J Respir Cell Mol Biol* 2000;22:45-50.
44. Kelley TJ, Elmer HL. In vivo alterations of IFN regulatory factor-1 and PIAS1 protein levels in cystic fibrosis epithelium. *J Clin Invest* 2000;106:403-410.
45. Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DW. Early pulmonary inflammation in infants with cystic fibrosis. *Am J Respir Crit Care Med* 1995;151:1075-1082.
46. Konstan MW, Berger M. Current understanding of the inflammatory process in cystic fibrosis: onset and etiology. *Pediatr Pulmonol* 1997;24:137-142.
47. Balfour-Lynn IM, Lavery A, Dinwiddie R. Reduced upper airway nitric oxide in cystic fibrosis. *Arch Dis Child* 1996;75:319-322.
48. Grasemann H, Michler E, Wallot M, Ratjen F. Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. *Pediatr Pulmonol* 1997;24:173-177.
49. Chmiel JF, Konstan MW, Saadane A, Krenicky JE, Lester KH, Berger M. Prolonged inflammatory response to acute *Pseudomonas* challenge in interleukin-10 knockout mice. *Am J Respir Crit Care Med* 2002;165:1176-1181.
50. Chmiel JF, Konstan MW, Knesebeck JE, Hilliard JB, Bonfield TL, Dawson DV, Berger M. IL-10 attenuates excessive inflammation in chronic *Pseudomonas* infection in mice. *Am J Respir Crit Care Med* 1999;160:2040-2047.
51. Ollero M, Junaidei O, Zaman MM, Tzamelis I, Ferrando AA, Andersson C, Blanco PG, Bialecki E, Freedman SD. Decreased expression of peroxisome proliferator activated receptor gamma in Cftr(-/-) mice. *J Cell Physiol* 2004;200:235-244.
52. Green S. PPAR: a mediator of peroxisome proliferator action. *Mutat Res* 1995;333:101-109.
53. Vanden Berghe W, Vermeulen L, Delerive P, De Bosscher K, Staels B, Haegeman G. A paradigm for gene regulation: inflammation, NF-kappaB and PPAR. *Adv Exp Med Biol* 2003;544:181-196.
54. Davis PB, Gupta S, Eastman J, Konstan MW. Inhibition of proinflammatory cytokine production by PPARgamma agonists in airway epithelial cells [abstract]. *Pediatr Pulmonol Suppl* 2003;25:A246.
55. Konstan MW, Walenga RW, Hilliard KA, Hilliard JB. Leukotriene B4 markedly elevated in the epithelial lining fluid of patients with cystic fibrosis. *Am Rev Respir Dis* 1993;148:896-901.
56. Meyer KC, Zimmerman J. Neutrophil mediators, *Pseudomonas*, and pulmonary dysfunction in cystic fibrosis. *J Lab Clin Med* 1993;121:654-661.
57. Birrer P, McElvaney NG, Rudeberg A, Sommer CW, Liechti-Gallati S, Kraemer R, Hubbard R, Crystal RG. Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. *Am J Respir Crit Care Med* 1994;150:207-213.
58. Konstan MW, Hilliard KA, Norvell TM, Berger M. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am J Respir Crit Care Med* 1994;150:448-454.
59. Armstrong DS, Grimwood K, Carzino R, Carlin JB, Olinsky A, Phelan PD. Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. *BMJ* 1995;310:1571-1572.
60. Noah TL, Black HR, Cheng PW, Wood RE, Leigh MW. Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. *J Infect Dis* 1997;175:638-647.
61. Muhlebach MS, Stewart PW, Leigh MW, Noah TL. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. *Am J Respir Crit Care Med* 1999;160:186-191.
62. Rosenfeld M, Gibson RL, McNamara S, Emerson J, Burns JL, Castile R, Hiatt P, McCoy K, Wilson CB, Inglis A, et al. Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. *Pediatr Pulmonol* 2001;32:356-366.
63. Meyer KC, Lewandoski JR, Zimmerman JJ, Nunley D, Calhoun WJ, Dopico GA. Human neutrophil elastase and elastase/alpha 1-antiprotease complex in cystic fibrosis: comparison with interstitial lung disease and evaluation of the effect of intravenously administered antibiotic therapy. *Am Rev Respir Dis* 1991;144:580-585.
64. McElvaney NG, Hubbard RC, Birrer P, Chernick MS, Caplan DB, Frank MM, Crystal RG. Aerosol alpha 1-antitrypsin treatment for cystic fibrosis. *Lancet* 1991;337:392-394.
65. McElvaney NG, Nakamura H, Birrer P, Hebert CA, Wong WL, Alphonso M, Baker JB, Catalano MA, Crystal RG. Modulation of airway inflammation in cystic fibrosis: in vivo suppression of interleukin-8 levels on the respiratory epithelial surface by aerosolization of recombinant secretory leukoprotease inhibitor. *J Clin Invest* 1992;90:1296-1301.
66. Wagener JS, Rock MJ, McCubbin MM, Hamilton SD, Johnson CA, Ahrens RC. Aerosol delivery and safety of recombinant human deoxyribonuclease in young children with cystic fibrosis: a bronchoscopic study. Pulmozyme Pediatric Bronchoscopy Study Group. *J Pediatr* 1998;133:486-491.
67. Roum JH, Borok Z, McElvaney NG, Grimes GJ, Bokser AD, Buhl R, Crystal RG. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. *J Appl Physiol* 1999;87:438-443.
68. Rosenfeld M, Gibson R, McNamara S, Emerson J, McCoy KS, Shell R, Borowitz D, Konstan MW, Retsch-Bogart G, Wilmott RW, et al. Serum and lower respiratory tract drug concentrations after tobramycin inhalation in young children with cystic fibrosis. *J Pediatr* 2001;139:572-577.
69. Gibson RL, Emerson J, McNamara S, Burns JL, Rosenfeld M, Yunker A, Hamblett N, Accurso F, Dovey M, Hiatt P, et al. Significant microbiological effect of inhaled tobramycin in young children with cystic fibrosis. *Am J Respir Crit Care Med* 2003;167:841-849.
70. Paul K, Rietschel E, Ballmann M, Griese M, Worlitzsch D, Shute J, Chen C, Schink T, Doring G, Van Koningsbruggen S, et al. Effect of treatment with dornase alpha on airway inflammation in patients with cystic fibrosis. *Am J Respir Crit Care Med* 2004;169:719-725.
71. Ratjen F, Paul K, Van Koningsbruggen S, Breitenstein S, Rietschel E, Nikolaizik W. DNA concentrations in BAL fluid of cystic fibrosis

- patients with early lung disease: influence of treatment with dornase alpha. *Pediatr Pulmonol* 2005;39:1-4.
72. Hilliard JB, Konstan MW, Davis PB. Inflammatory mediators in CF patients. In: Skach WR, editor. *Cystic fibrosis methods and protocols*. Totowa, NJ: Humana Press; 2002. pp. 409-432.
 73. Davis SD, Brody AS, Emond MJ, Brumback LC, Rosenfeld M. End-points for clinical trials in young children with cystic fibrosis. *Proc Am Thorac Soc* 2007;4:418-430.
 74. Cunningham S, McColm JR, Ho LP, Greening AP, Marshall TG. Measurement of inflammatory markers in the breath condensate of children with cystic fibrosis. *Eur Respir J* 2000;15:955-957.
 75. Carpagnano GE, Barnes PJ, Geddes DM, Hodson ME, Kharitonov SA. Increased leukotriene B4 and interleukin-6 in exhaled breath condensate in cystic fibrosis. *Am J Respir Crit Care Med* 2003;167:1109-1112.
 76. Carpagnano GE, Barnes PJ, Francis J, Wilson N, Bush A, Kharitonov SA. Breath condensate pH in children with cystic fibrosis and asthma: a new noninvasive marker of airway inflammation? *Chest* 2004;125:2005-2010.
 77. Bodini A, D'Orazio C, Peroni D, Corradi M, Folesani G, Baraldi E, Assael BM, Boner A, Piacentini GL. Biomarkers of neutrophilic inflammation in exhaled air of cystic fibrosis children with bacterial airway infections. *Pediatr Pulmonol* 2005;40:494-499.
 78. Robroeks CM, Jobsis Q, Damoiseaux JG, Heijmans PH, Rosias PP, Hendriks HJ, Dompeling E. Cytokines in exhaled breath condensate of children with asthma and cystic fibrosis. *Ann Allergy Asthma Immunol* 2006;96:349-355.
 79. Celio S, Troxler H, Durka SS, Chladek J, Wildhaber JH, Sennhauser FH, Heizmann CW, Moeller A. Free 3-nitrotyrosine in exhaled breath condensates of children fails as a marker for oxidative stress in stable cystic fibrosis and asthma. *Nitric Oxide* 2006;15:226-232.
 80. Rosias PP, Den Hartog GJ, Robroeks CM, Bast A, Donckerwolcke RA, Heynens JW, Suykerbuyk J, Hendriks HJ, Jobsis Q, Dompeling E. Free radicals in exhaled breath condensate in cystic fibrosis and healthy subjects. *Free Radic Res* 2006;40:901-909.
 81. Johansen HK, Norregaard L, Gotzsche PC, Pressler T, Koch C, Hoiby N. Antibody response to *Pseudomonas aeruginosa* in cystic fibrosis patients: a marker of therapeutic success? A 30-year cohort study of survival in Danish CF patients after onset of chronic *P. aeruginosa* lung infection. *Pediatr Pulmonol* 2004;37:427-432.
 82. Li Z, Kosorok MR, Farrell PM, Laxova A, West SE, Green CG, Collins J, Rock MJ, Splaingard ML. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. *JAMA* 2005;293:581-588.
 83. Ratjen F, Walter H, Haug M, Meisner C, Grasemann H, Doring G. Diagnostic value of serum antibodies in early *Pseudomonas aeruginosa* infection in cystic fibrosis patients. *Pediatr Pulmonol* 2007;42:249-255.
 84. Dorlochter L, Carlsson M, Olafsdottir EJ, Roksund OD, Rosendahl K, Fluge G. Anti-neutrophil cytoplasmic antibodies and lung disease in cystic fibrosis. *J Cyst Fibros* 2004;3:179-183.
 85. Downey DG, Martin SL, Dempster M, Moore JE, Keogan MT, Starcher B, Edgar J, Bilton D, Elborn JS. The relationship of clinical and inflammatory markers to outcome in stable patients with cystic fibrosis. *Pediatr Pulmonol* 2007;42:216-220.
 86. Levy H, Kalish LA, Huntington I, Weller N, Gerard C, Silverman EK, Celedon JC, Pier GB, Weiss ST. Inflammatory markers of lung disease in adult patients with cystic fibrosis. *Pediatr Pulmonol* 2007;42:256-262.
 87. Brown MA, Morgan WJ, Finley PR, Scuderi P. Circulating levels of tumor necrosis factor and interleukin-1 in cystic fibrosis. *Pediatr Pulmonol* 1991;10:86-91.
 88. Norman D, Elborn JS, Cordon SM, Rayner RJ, Wiseman MS, Hiller EJ, Shale DJ. Plasma tumour necrosis factor alpha in cystic fibrosis. *Thorax* 1991;46:91-95.
 89. Dean TP, Dai Y, Shute JK, Church MK, Warner JO. Interleukin-8 concentrations are elevated in bronchoalveolar lavage, sputum, and sera of children with cystic fibrosis. *Pediatr Res* 1993;34:159-161.
 90. Greally P, Hussain MJ, Vergani D, Price JF. Serum interleukin-1 alpha and soluble interleukin-2 receptor concentrations in cystic fibrosis. *Arch Dis Child* 1993;68:785-787.
 91. Kronborg G, Hansen MB, Svenson M, Fomsgaard A, Hoiby N, Bendtzen K. Cytokines in sputum and serum from patients with cystic fibrosis and chronic *Pseudomonas aeruginosa* infection as markers of destructive inflammation in the lungs. *Pediatr Pulmonol* 1993;15:292-297.
 92. Wilmott RW, Frenze M, Kociela V, Peng L. Plasma interleukin-1 alpha and beta, tumor necrosis factor-alpha, and lipopolysaccharide concentrations during pulmonary exacerbations of cystic fibrosis. *Pediatr Pulmonol* 1994;18:21-27.
 93. McColley SA, Stellmach V, Boas SR, Jain M, Crawford SE. Serum vascular endothelial growth factor is elevated in cystic fibrosis and decreases with treatment of acute pulmonary exacerbation. *Am J Respir Crit Care Med* 2000;161:1877-1880.
 94. Augarten A, Paret G, Ayneri I, Akons H, Aviram M, Bentur L, Blau H, Efrati O, Szeinberg A, Barak A, et al. Systemic inflammatory mediators and cystic fibrosis genotype. *Clin Exp Med* 2004;4:99-102.
 95. Schmitt-Grohe S, Naujoks C, Bargon J, Wagner TO, Schubert R, Hippe V, Zielen S. Interleukin-8 in whole blood and clinical status in cystic fibrosis. *Cytokine* 2005;29:18-23.
 96. Moser C, Jensen PO, Pressler T, Frederiksen B, Lanng S, Kharazmi A, Koch C, Hoiby N. Serum concentrations of GM-CSF and G-CSF correlate with the Th1/Th2 cytokine response in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. *APMIS* 2005;113:400-409.
 97. Ionescu AA, Mickleborough TD, Bolton CE, Lindley MR, Nixon LS, Dunseath G, Luzio S, Owens DR, Shale DJ. The systemic inflammatory response to exercise in adults with cystic fibrosis. *J Cyst Fibros* 2006;5:105-112.
 98. Elborn JS, Norman D, Delamere FM, Shale DJ. In vitro tumor necrosis factor- α secretion by monocytes from patients with cystic fibrosis. *Am J Respir Cell Mol Biol* 1992;6:207-211.
 99. Hauber HP, Gholami D, Koppermann G, Heuer HE, Meyer A, Pforte A. Increased expression of interleukin-13 but not interleukin-4 in cystic fibrosis patients. *J Cyst Fibros* 2003;2:189-194.
 100. Corvol H, Fitting C, Chadelat K, Jacquot J, Tabary O, Boule M, Cavaillon JM, Clement A. Distinct cytokine production by lung and blood neutrophils from children with cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L997-1003.
 101. Hauber HP, Beyer IS, Meyer A, Pforte A. Decreased interleukin-18 expression in BAL cells and peripheral blood mononuclear cells in adult cystic fibrosis patients. *J Cyst Fibros* 2004;3:129-131.
 102. Zaman MM, Gelrud A, Junaidi O, Regan MM, Warny M, Shea JC, Kelly C, O'Sullivan BP, Freedman SD. Interleukin 8 secretion from monocytes of subjects heterozygous for the deltaF508 cystic fibrosis transmembrane conductance regulator gene mutation is altered. *Clin Diagn Lab Immunol* 2004;11:819-824.
 103. Brazova J, Sediva A, Pospisilova D, Vavrova V, Pohunek P, Macek M Jr, Bartunkova J, Lauschmann H. Differential cytokine profile in children with cystic fibrosis. *Clin Immunol* 2005;115:210-215.
 104. Rayner RJ, Wiseman MS, Cordon SM, Norman D, Hiller EJ, Shale DJ. Inflammatory markers in cystic fibrosis. *Respir Med* 1991;85:139-145.
 105. Nixon LS, Yung B, Bell SC, Elborn JS, Shale DJ. Circulating immunoreactive interleukin-6 in cystic fibrosis. *Am J Respir Crit Care Med* 1998;157:1764-1769.
 106. Stone PJ, Konstan MW, Berger M, Dorkin HL, Franzblau C, Snider GL. Elastin and collagen degradation products in urine of patients with cystic fibrosis. *Am J Respir Crit Care Med* 1995;152:157-162.
 107. Sampson AP, Spencer DA, Green CP, Piper PJ, Price JF. Leukotrienes in the sputum and urine of cystic fibrosis children. *Br J Clin Pharmacol* 1990;30:861-869.
 108. Bruce MC, Poncz L, Klinger JD, Stern RC, Tomashefski JF Jr, Dearborn DG. Biochemical and pathologic evidence for proteolytic destruction of lung connective tissue in cystic fibrosis. *Am Rev Respir Dis* 1985;132:529-535.
 109. Smith AL, Redding G, Doershuk C, Goldmann D, Gore E, Hilman B, Marks M, Moss R, Ramsey B, Rubio T, et al. Sputum changes associated with therapy for endobronchial exacerbation in cystic fibrosis. *J Pediatr* 1988;112:547-554.
 110. Richman-Eisenstat JB, Jorens PG, Hebert CA, Ueki I, Nadel JA. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. *Am J Physiol* 1993;264:L413-L418.
 111. Greally P, Hussein MJ, Cook AJ, Sampson AP, Piper PJ, Price JF. Sputum tumour necrosis factor-alpha and leukotriene concentrations in cystic fibrosis. *Arch Dis Child* 1993;68:389-392.
 112. Regelman WE, Siefferman CM, Herron JM, Elliott GR, Clawson CC, Gray BH. Sputum peroxidase activity correlates with the severity of lung disease in cystic fibrosis. *Pediatr Pulmonol* 1995;19:1-9.
 113. Koller DY, Nething I, Otto J, Urbanek R, Eichler I. Cytokine concentrations in sputum from patients with cystic fibrosis and their relation to eosinophil activity. *Am J Respir Crit Care Med* 1997;155:1050-1054.

114. Wolter JM, Rodwell RL, Bowler SD, McCormack JG. Cytokines and inflammatory mediators do not indicate acute infection in cystic fibrosis. *Clin Diagn Lab Immunol* 1999;6:260-265.
115. Pukhalsky AL, Kapranov NI, Kalashnikova EA, Shmarina GV, Shabalova LA, Kokorovtseva SN, Pukhalskaya DA, Kashirskaja NJ, Simonova OI. Inflammatory markers in cystic fibrosis patients with lung *Pseudomonas aeruginosa* infection. *Mediators Inflamm* 1999;8:159-167.
116. Karpati F, Hjelte FL, Wretling B. TNF-alpha and IL-8 in consecutive sputum samples from cystic fibrosis patients during antibiotic treatment. *Scand J Infect Dis* 2000;32:75-79.
117. Sagel SD, Kapsner R, Osberg I, Sontag MK, Accurso FJ. Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. *Am J Respir Crit Care Med* 2001;164:1425-1431.
118. Ordonez CL, Henig NR, Mayer-Hamblett N, Accurso FJ, Burns JL, Chmiel JF, Daines CL, Gibson RL, McNamara S, Retsch-Bogart GZ, et al. Inflammatory and microbiologic markers in induced sputum after intravenous antibiotics in cystic fibrosis. *Am J Respir Crit Care Med* 2003;168:1471-1475.
119. Thomassen MJ, Klinger JD, Badger SJ, van Heeckeren DW, Stern RC. Cultures of thoracotomy specimens confirm usefulness of sputum cultures in cystic fibrosis. *J Pediatr* 1984;104:352-356.
120. Gilljam H, Malmberg AS, Strandvik B. Conformity of bacterial growth in sputum and contamination free endobronchial samples in patients with cystic fibrosis. *Thorax* 1986;41:641-646.
121. Aaron SD, Kottachchi D, Ferris WJ, Vandemheen KL, St Denis ML, Plouffe A, Doucette SP, Saginur R, Chan FT, Ramotar K. Sputum versus bronchoscopy for diagnosis of *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Eur Respir J* 2004;24:631-637.
122. van Belkum A, Renders NH, Smith S, Overbeek SE, Verbrugh HA. Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients. *FEMS Immunol Med Microbiol* 2000;27:51-57.
123. Kolak M, Karpati F, Monstein HJ, Jonasson J. Molecular typing of the bacterial flora in sputum of cystic fibrosis patients. *Int J Med Microbiol* 2003;293:309-317.
124. Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* 2003;41:3548-3558.
125. Pedersen SK, Sloane AJ, Prasad SS, Sebastian LT, Lindner RA, Hsu M, Robinson M, Bye PT, Weinberger RP, Harry JL. An immunoproteomic approach for identification of clinical biomarkers for monitoring disease: application to cystic fibrosis. *Mol Cell Proteomics* 2005;4:1052-1060.
126. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. *J Pediatr* 2002;141:811-817.
127. Dakin CJ, Pereira JK, Henry RL, Wang H, Morton JR. Relationship between sputum inflammatory markers, lung function, and lung pathology on high-resolution computed tomography in children with cystic fibrosis. *Pediatr Pulmonol* 2002;33:475-482.
128. Sagel SD, Kapsner RK, Osberg I. Induced sputum matrix metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. *Pediatr Pulmonol* 2005;39:224-232.
129. Kim JS, Okamoto K, Rubin BK. Pulmonary function is negatively correlated with sputum inflammatory markers and cough clearability in subjects with cystic fibrosis but not those with chronic bronchitis. *Chest* 2006;129:1148-1154.
130. Mayer-Hamblett N, Aitken ML, Accurso FJ, Kronmal RA, Konstan MW, Burns JL, Sagel SD, Ramsey BW. Association between pulmonary function and sputum biomarkers in cystic fibrosis. *Am J Respir Crit Care Med* 2007;175:822-828.
131. Salva PS, Doyle NA, Graham L, Eigen H, Doerschuk CM. TNF-alpha, IL-8, soluble ICAM-1, and neutrophils in sputum of cystic fibrosis patients. *Pediatr Pulmonol* 1996;21:11-19.
132. Schuster A, Haarmann A, Wahn V. Cytokines in neutrophil-dominated airway inflammation in patients with cystic fibrosis. *Eur Arch Otorhinolaryngol* 1995;252:S59-S60.
133. Francoeur C, Denis M. Nitric oxide and interleukin-8 as inflammatory components of cystic fibrosis. *Inflammation* 1995;19:587-598.
134. Cunningham S, McCollm JR, Mallinson A, Boyd I, Marshall TG. Duration of effect of intravenous antibiotics on spirometry and sputum cytokines in children with cystic fibrosis. *Pediatr Pulmonol* 2003;36:43-48.
135. Murray TS, Egan M, Kazmierczak BI. *Pseudomonas aeruginosa* chronic colonization in cystic fibrosis patients. *Curr Opin Pediatr* 2007;19:83-88.
136. Shah PL, Scott SF, Knight RA, Hodson ME. The effects of recombinant human DNase on neutrophil elastase activity and interleukin-8 levels in the sputum of patients with cystic fibrosis. *Eur Respir J* 1996;9:531-534.
137. Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, Coquillette S, Fieberg AY, Accurso FJ, Campbell PW III. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA* 2003;290:1749-1756.
138. Reinhardt N, Chen CI, Loppow D, Schink T, Kleinau I, Jorres RA, Wahn U, Magnussen H, Paul KP. Cellular profiles of induced sputum in children with stable cystic fibrosis: comparison With BAL. *Eur Respir J* 2003;22:497-502.
139. Smountas AA, Lands LC, Mohammed SR, Grey V. Induced sputum in cystic fibrosis: within-week reproducibility of inflammatory markers. *Clin Biochem* 2004;37:1031-1036.
140. Ordonez CL, Kartashov AI, Wohl ME. Variability of markers of inflammation and infection in induced sputum in children with cystic fibrosis. *J Pediatr* 2004;145:689-692.
141. Meyer KC, Sharma A. Regional variability of lung inflammation in cystic fibrosis. *Am J Respir Crit Care Med* 1997;156:1536-1540.
142. Efthimiadis A, Pizzichini MM, Pizzichini E, Dolovich J, Hargreave FE. Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol vs phosphate-buffered saline. *Eur Respir J* 1997;10:1336-1340.
143. Louis R, Shute J, Goldring K, Perks B, Lau LC, Radermecker M, Djukanovic R. The effect of processing on inflammatory markers in induced sputum. *Eur Respir J* 1999;13:660-667.
144. Grebski E, Peterson C, Medici TC. Effect of physical and chemical methods of homogenization on inflammatory mediators in sputum of asthma patients. *Chest* 2001;119:1521-1525.
145. Kim JS, Hackley GH, Okamoto K, Rubin BK. Sputum processing for evaluation of inflammatory mediators. *Pediatr Pulmonol* 2001;32:152-158.
146. Woolhouse IS, Bayley DL, Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. *Thorax* 2002;57:667-671.
147. Kelly MM, Keatings V, Leigh R, Peterson C, Shute J, Venge P, Djukanovic R. Analysis of fluid-phase mediators. *Eur Respir J Suppl* 2002;37:24s-39s.
148. Hilliard J, Minillo B, Krenicky J, Pallotta S, Konstan M. Sputum in cystic fibrosis: preliminary evaluation of in vitro digestive methods using representative outcome measures [abstract]. *Pediatr Pulmonol Suppl* 2004;27:271.
149. Gibson PG, Girgis-Gabardo A, Morris MM, Mattoli S, Kay JM, Dolovich J, Denburg J, Hargreave FE. Cellular characteristics of sputum from patients with asthma and chronic bronchitis. *Thorax* 1989;44:693-699.
150. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992;47:25-29.
151. Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993;147:1126-1131.
152. Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995;95:843-852.
153. Henry RL, Gibson PG, Carty K, Cai Y, Francis JL. Airway inflammation after treatment with aerosolized deoxyribonuclease in cystic fibrosis. *Pediatr Pulmonol* 1998;26:97-100.
154. Suri R, Marshall LJ, Wallis C, Metcalfe C, Bush A, Shute JK. Effects of recombinant human DNase and hypertonic saline on airway inflammation in children with cystic fibrosis. *Am J Respir Crit Care Med* 2002;166:352-355.
155. Equi A, Balfour-Lynn IM, Bush A, Rosenthal M. Long term azithromycin in children with cystic fibrosis: a randomised, placebo-controlled crossover trial. *Lancet* 2002;360:978-984.
156. Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D, Milla C, Brody AS, Clancy JP, Ramsey B, et al. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic

- fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* 2004;125:509–521.
157. Moss RB, Mayer-Hamblett N, Wagener J, Daines C, Hale K, Ahrens R, Gibson RL, Anderson P, Retsch-Bogart G, Nasr SZ, *et al.* Randomized, double-blind, placebo-controlled, dose-escalating study of aerosolized interferon gamma-1b in patients with mild to moderate cystic fibrosis lung disease. *Pediatr Pulmonol* 2005;39:209–218.
158. Husson MO, Wizla-Derambure N, Turck D, Gosset P, Wallaert B. Effect of intermittent inhaled tobramycin on sputum cytokine profiles in cystic fibrosis. *J Antimicrob Chemother* 2005;56:247–249.
159. Colombo C, Costantini D, Rocchi A, Cariani L, Garlaschi ML, Tirelli S, Calori G, Copreni E, Conese M. Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy. *Pediatr Pulmonol* 2005;40:15–21.
160. Stelmach I, Korzeniewska A, Stelmach W, Majak P, Grzelewski T, Jerzynska J. Effects of montelukast treatment on clinical and inflammatory variables in patients with cystic fibrosis. *Ann Allergy Asthma Immunol* 2005;95:372–380.
161. Martin SL, Downey D, Bilton D, Keogan MT, Edgar J, Elborn JS. Safety and efficacy of recombinant alpha(1)-antitrypsin therapy in cystic fibrosis. *Pediatr Pulmonol* 2006;41:177–183.
162. Tirouvanziam R, Conrad CK, Bottiglieri T, Herzenberg LA, Moss RB, Herzenberg LA. High-dose oral N-acetylcysteine, a glutathione pro-drug, modulates inflammation in cystic fibrosis. *Proc Natl Acad Sci USA* 2006;103:4628–4633.
163. Konstan MW, Krenicky JE, Finney MR, Kirchner HL, Hilliard KA, Hilliard JB, Davis PB, Hoppel CL. Effect of ibuprofen on neutrophil migration in vivo in cystic fibrosis and healthy subjects. *J Pharmacol Exp Ther* 2003;306:1086–1091.
164. Suri R, Marshall LJ, Wallis C, Metcalfe C, Shute JK, Bush A. Safety and use of sputum induction in children with cystic fibrosis. *Pediatr Pulmonol* 2003;35:309–313.
165. Nightingale JA, Rogers DF, Barnes PJ. Effect of repeated sputum induction on cell counts in normal volunteers. *Thorax* 1998;53:87–90.
166. Holz O, Richter K, Jorres RA, Speckin P, Mucke M, Magnussen H. Changes in sputum composition between two inductions performed on consecutive days. *Thorax* 1998;53:83–86.
167. Paggiaro PL, Chanez P, Holz O, Ind PW, Djukanovic R, Maestrelli P, Sterk PJ. Sputum induction. *Eur Respir J Suppl* 2002;37:3s–8s.
168. Efthimiadis A, Spanevello A, Hamid Q, Kelly MM, Linden M, Louis R, Pizzichini MM, Pizzichini E, Ronchi C, Van Overvel F, *et al.* Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. *Eur Respir J Suppl* 2002;37:19s–23s.
169. Tirelli AS, Colombo C, Torresani E, Cariani L, Arnaboldi E, Conese M. Validation of an automated sensitive immunoassay for quantitation of cytokines in the sputum of cystic fibrosis patients. *Clin Chem Lab Med* 2007;45:108–111.
170. Cummings DM, King DE, Mainous AG, Geesey ME. Combining serum biomarkers: the association of C-reactive protein, insulin sensitivity, and homocysteine with cardiovascular disease history in the general US population. *Eur J Cardiovasc Prev Rehabil* 2006;13:180–185.
171. Sabatine MS, Morrow DA, de Lemos JA, Gibson CM, Murphy SA, Rifai N, McCabe C, Antman EM, Cannon CP, Braunwald E. Multimarker approach to risk stratification in non-ST elevation acute coronary syndromes: simultaneous assessment of troponin I, C-reactive protein, and B-type natriuretic peptide. *Circulation* 2002;105:1760–1763.