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Neutrophil elastase correlates with increased sphingolipid content in cystic fibrosis sputum

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Abstract

Introduction: Sphingolipids are associated with the regulation of pulmonary inflammation. Although sphingolipids have been investigated in the context of cystic fibrosis (CF), the focus has been on loss of CF transmembrane conductance regulator (CFTR) function in mice, and in CF human lung epithelial cell lines. The sphingolipid content of CF sputum and the potential link between ceramide and airway inflammation in CF remain relatively unexplored.

Methods: Fifteen patients with CF provided two spontaneously expectorated sputum samples, one collected during a hospitalization for an acute pulmonary exacerbation and one from an outpatient visit at a time of clinical stability. Sputum was processed, and the supernatant assessed for active neutrophil elastase (NE) using a chromogenic microplate assay and sphingolipid content using reverse phase high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). Relevant demographic data including age, sex, CF genotype, FEV₁ % predicted, and sputum bacteriology were assessed as possible modifying factors that could influence the correlation between NE and sputum sphingolipids. Data were analyzed for linear correlation, with statistical significance pre-defined as $P < 0.05$.

Results: There was a significant association between the concentration of active NE and ceramide, sphingomyelin, and monohexosylceramide moieties as well as sphingosine-1-phosphate. The presence of Methicillin-resistant *Staphylococcus aureus* (MRSA), FEV₁ % predicted, and female gender further strengthened the association of NE and sphingolipids, but *Pseudomonas aeruginosa* had no effect on the association between NE and sphingolipids.

Conclusions: These data suggest that NE may increase pro-inflammatory sphingolipid signaling, and the association is strengthened in female patients and patients with MRSA.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Keywords

ceramide; inflammation; lung disease

1 | INTRODUCTION

Despite advances in medicine over the past decades, CF remains a debilitating, life-shortening disease. While many organ systems are affected by CF, the most common cause of patient morbidity and mortality is lung disease, which is characterized by recurring cycles of infection and neutrophilic inflammation.¹ Patients' airways contain high concentrations of proteolytic enzymes, such as neutrophil elastase (NE), a biomarker for lung disease progression in CF. Patients with CF can have up to micromolar concentrations of NE in their airways, and this protease further stimulates an inflammatory response.² The cycle of infection and over-exuberant inflammation damages the lungs, resulting in gradual decrements in lung function and eventually resulting in respiratory failure.

Bioactive lipids, such as sphingolipids, can act as signaling molecules; the sphingolipids ceramide, ceramide-1-phosphate, and sphingosine-1-phosphate in particular have been implicated as modulators of pulmonary inflammation, with ceramide and ceramide-1-phosphate being regarded as pro-inflammatory.^{3–6} Studies of sphingolipid imbalances in CF have focused on changes that result from CF transmembrane conductance regulator (CFTR) dysfunction.^{7,8} However, Brodlie et al⁹ demonstrated that d_{18:1/16:0}, d_{18:1/18:0} and d_{18:1/20:0} ceramides are increased in the lower airways of patients with CF, and that increased ceramide is associated with greater numbers of neutrophils. Thus, there is a correlation between increased tissue ceramide and neutrophilic inflammation.⁹

We have previously demonstrated, using a mouse model of NE-induced inflammation,^{10–12} that NE increases the concentration of ceramide in murine airways, due to increases in d_{18:1/22:0}, d_{18:1/24:1}, and d_{18:1/24:0} ceramide moieties.¹³ This increase in ceramide is associated with a concurrent increase in serine palmitoyltransferase (SPT) long chain 2, a subunit of the enzyme that catalyzes a rate-limiting step in de novo sphingolipid synthesis, and upregulation of d_{18:1/22:0} and d_{18:1/24:1} ceramide can be blocked using an SPT inhibitor, such as myriocin.¹³ Administering myriocin to NE-treated animals also decreases pro-inflammatory signals, including CXCL1/keratinocyte chemoattractant (KC, the mouse homologue of IL8) and high mobility group box 1 (HMGB1), though it does not affect the total white blood cell count or percent of neutrophils present in murine bronchoalveolar lavage.¹³

There are few studies describing the sphingolipid content present in bronchoalveolar lavage or sputum from patients with CF. Analysis of CF sputum identified elevated sphingomyelin and ceramide compared to control samples gathered from healthy donors, with no apparent correlation to the presence of any bacterial genera.^{14,15} In a longitudinal study of one individual with CF, during four pulmonary exacerbations, sputum ceramide (d_{18:1/16:0}) was significantly increased, suggesting an association between sputum ceramide levels and airway inflammation.¹⁵ To the extent of our knowledge, there are currently no studies that evaluate whether human NE or other inflammatory mediators correlate with ceramide levels

in sputum. We hypothesized that an increased level of ceramide in sputum from patients with CF would correlate with increasing concentrations of active NE.

2 | MATERIALS AND METHODS

2.1 | Subject recruitment, sputum sample collection

We performed a retrospective study of 15 subjects with CF, to analyze at least two sputum samples per subject, one collected during a hospitalization for pulmonary exacerbation and a second sample collected during an outpatient visit at a time of clinical stability, to correlate the sputum levels of active NE with sputum sphingolipid content and with lung function (FEV₁ % predicted). Participants were recruited from the Adult and Pediatric CF Centers at VCU Medical Center. The study was approved by the Institutional Review Board (IRB) at VCU; subjects or parents/guardians provided written informed consent before enrollment into the VCU Biospecimen Repository and for participation in the CF Foundation (CFF) Registry. All subjects spontaneously expectorated sputum and had mild-to-moderate (FEV₁ 50–98% predicted) or severe (FEV₁ < 50% predicted) obstructive lung disease. The time interval between samples acquired during a hospitalization for pulmonary exacerbation and samples collected during an outpatient visit was 6 months. Samples collected during hospitalization were gathered between 1 and 9 days of admission with CF pulmonary exacerbation, with the median day of sampling being day 1. Sputa collected on the day patients were advised to be admitted were also labeled as day 1 of hospitalization. An aliquot of sputum was sent for bacteriology cultures, performed according to CFF standard protocols at the beginning of therapy.¹⁶ The remaining aliquots of sputum were visually separated from saliva and oral detritus, and stored for later analysis at –80°C.

2.2 | Subject demographics

Mean subject age was 20 years, median subject age was 18 years, and the age range was 12–39 years of age. Eleven out of 15 patients were followed in Pediatric CF Center because they were less than 21 years of age. Seven out of fifteen (46.7%) of the subjects were female. Six of 15 (40%) subjects were homozygous for F508del, eight of 15 (53.3%) were heterozygous for F508del, and one subject had an unknown CF genotype. One out of fifteen (~6.7%) subjects had mild obstructive lung disease (FEV₁ % predicted >80%), five of 15 (~33.3%) had moderate obstructive lung disease (FEV₁ % predicted 50% and <80%), and nine out of 15 (60%) subjects had severe obstructive lung disease (FEV₁ % predicted <50%). Analysis of the bacteriology cultures revealed that approximately 46.7% of sputum samples were positive for methicillin-resistant *Staphylococcus aureus* (MRSA) and about 53.3% of sputum samples were positive for *Pseudomonas aeruginosa*.

2.3 | Sputum processing

Frozen sputa were thawed on ice. Thawed sputum samples were weighed, and mixed with an equal (1:1, W:V) volume of normal saline with 10% Sputolysin (0.1% DTT; Calbiochem/EMD Millipore, Billerica, MA). Diluted sputum samples were mixed by vortex at room temperature for 15–30 s and incubated at 37°C for 15 min. Next, samples were mixed gently by inverting, and centrifuged at 25 000g for 30 min at 4°C to collect the

supernatant. The supernatant was immediately aliquoted and used for measuring sphingolipid levels and NE activity.

2.4 | NE activity assay

NE activity in supernatants was measured using a chromogenic microtiter plate assay with Succinyl-Ala-Ala-Pro-Val-p-nitroanalide (3 mM, in 50% DMSO; Sigma-Aldrich, St. Louis, MO) as the substrate, as detailed previously,^{17,18} which is the standard method for analyzing the levels of NE in both CF sputum and BAL.^{19–21} Active NE was expressed in nM, extrapolating the concentration from a standard curve using human sputum-derived active NE (SE563, Elastin Products, Owensville, MI). This assay can detect proteolytically active NE, but not NE complexed with alpha-1-antitrypsin (A1AT), as the NE-A1AT interaction results in an irreversible inactivation of NE.

2.5 | Sphingolipid analysis

Sputum supernatant was assessed for sphingolipid content (sphingosine, sphinganine, sphingosine-1-phosphate, sphinganine-1-phosphate, sphingomyelin, ceramide, monohexosylceramide chain lengths d_{18:1/14:0}, d_{18:1/16:0}, d_{18:1/18:1}, d_{18:1/18:0}, d_{18:1/22:0}, d_{18:1/24:1}, d_{18:1/24:0}, d_{18:1/26:1}, and d_{18:1/26:0}) by the lipidomics/metabolomics core at VCU, using reverse phase, high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS).^{5,22}

2.6 | Statistical analyses

We used a two-tailed, nonparametric, paired statistical test: the Wilcoxon signed rank test, to determine whether there were significant differences between stable and exacerbated sputum measures for sphingolipid levels, the concentration of active NE, and FEV₁ % predicted using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).²³ Differences between groups were considered significant at $P < 0.05$.

We used linear correlation to assess the association between concentration of active NE and sphingolipid levels using GraphPad Prism 5 (GraphPad Software, Inc.). Data were analyzed using a linear mixed model,²⁴ to determine the likelihood of an association between the concentration of NE and various sphingolipids, as well as whether (i) bacterial culture positive for methicillin resistant *Staphylococcus aureus* (MRSA) or *Pseudomonas aeruginosa*; (ii) patient lung function; or (iii) patient gender, modified the association. A P -value less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Sputum sphingolipids increased during hospitalization for CF pulmonary exacerbation

Sphingolipid content varied among subjects (Supplementary E-Figure 1–4) but only d_{18:1/14:0} ceramide ($P = 0.0215$), d_{18:1/24:1} ceramide ($P = 0.0413$), and d_{18:1/24:0} monohexosylceramide ($P = 0.0256$) were significantly increased in sputa collected during hospitalization compared to samples collected during outpatient clinic visits (Figure 1C–E). As expected, lung function (FEV₁ % predicted) was significantly decreased during

hospitalization for pulmonary exacerbations compared to outpatient clinic visits for individuals ($P = 0.0182$) (Figure 1A). However, the sputum concentration of active NE collected during hospitalization for CF pulmonary exacerbations were not significantly different from samples collected during outpatient visits ($P = 0.4678$) (Figure 1B).

3.2 | Sphingolipids increased with elevated NE

We found statistically significant linear correlations between the concentrations of active NE and total sphingolipid moieties in subject sputa. Linear correlations were statistically significant between the concentrations of active NE and total sphingomyelin ($r^2 = 0.1977$; $P = 0.0138$) (Figure 2A); total ceramide ($r^2 = 0.1523$; $P = 0.033$) (Figure 2B); total monohexosylceramide ($r^2 = 0.2293$; $P = 0.0074$) (Figure 2C), and total sphingosine-1-phosphate (S1P) ($r^2 = 0.2570$; $P = 0.0042$) (Figure 2D).

Several different sphingomyelin moieties were increased as the levels of active NE were elevated. There was a statistically significant linear correlation between active NE and $d_{18:1/14:0}$ ($r^2 = 0.3641$; $P = 0.0004$), $d_{18:1/18:1}$ ($r^2 = 0.1555$; $P = 0.031$), $d_{18:1/22:0}$ ($r^2 = 0.2933$; $P = 0.0020$), $d_{18:1/24:1}$ ($r^2 = 0.2945$; $P = 0.0019$), and $d_{18:1/24:0}$ ($r^2 = 0.1406$; $P = 0.0412$) sphingomyelin (Figure 3). When assessing the association of active NE and ceramide of various chain lengths, we observed a statistically significant linear correlation between active NE and $d_{18:1/22:0}$ ceramide ($r^2 = 0.1478$; $P = 0.0359$) and $d_{18:1/24:0}$ ceramide ($r^2 = 0.2620$; $P = 0.0038$), as well as between active NE and two monohexosylceramide moieties— $d_{18:1/16:0}$ ($r^2 = 0.2725$; $P = 0.0031$) and $d_{18:1/24:0}$ ($r^2 = 0.1641$; $P = 0.0264$) (Figure 4).

3.3 | Modifying factors that influence the association between sphingolipids and NE in CF sputum

The presence of bacteria in sputum, severity of lung disease, and patient gender modified the association between active NE and certain sphingolipid moieties in CF sputum. Sputa that were positive for MRSA had significantly strengthened associations with $d_{18:1/14:0}$ sphingomyelin and $d_{18:1/24:0}$ ceramide, as well as total monohexosylceramide, due to changes in $d_{18:1/14:0}$ and $d_{18:1/16:0}$ monohexosylceramide (Table 1). The presence of *P. aeruginosa* in sputum did not significantly affect the associations between sphingolipids and active NE. FEV₁ (% predicted) affected the association between $d_{18:1/14:0}$ sphingomyelin and active NE and $d_{18:1/14:0}$ monohexosylceramide; the greater the lung function, the weaker the association between active NE and these two $d_{18:1/14:0}$ sphingolipid moieties (Table 1). Finally, the subject's gender significantly affected the association between $d_{18:1/14:0}$ sphingomyelin and active NE as well as $d_{18:1/14:0}$ monohexosylceramide and active NE. Sputa from females had a stronger association between those two sphingolipid moieties and active NE (Table 1).

4 | DISCUSSION

In this study, we report a novel observation, demonstrating a relationship between active NE and sphingolipids present in the sputum of patients with CF. Increasing concentrations of NE were associated with higher levels of a variety of sphingolipids in sputum; there were

statistically significant linear correlations between the levels of active NE and the concentrations of total sphingomyelin, ceramide, monohexosylceramide, and S1P.

While our focus has been inflammation, heretofore, the aberrant sphingolipid homeostasis observed in airway and nasal culture of human *Cftr*-deficient cells^{8,25,26} and *Cftr*-deficient mice,^{8,26,27} has been attributed to loss of CFTR function. However, examining sphingolipid biosynthesis in experiments with human airway epithelial cell lines has yielded contradictory results. Hamai et al²⁵ demonstrated that decreasing CFTR expression through genetic manipulation or expressing mutant F508delCFTR in airway epithelial cell lines results in increased intracellular sphingosine, sphinganine, and sphingomyelin, as well as d_{18:0/16:0}, d_{18:1/22:0}, d_{18:1/24:0}, and d_{18:1/26:0} ceramide, with concurrent decreases in d_{18:1/18:1} and d_{18:0/18:0} ceramide. Decreased expression of CFTR was also associated with increased protein SPT long chain 1, a subunit of the enzyme SPT, which is responsible for the rate-limiting step of de novo sphingolipid synthesis. In contrast, Yu et al²⁸ observed that decreased or mutant CFTR affects the function of acid sphingomyelinase (SMase), a key enzyme in a pathway that produces ceramide from sphingomyelin, in human airway epithelial cell lines, with *Cftr*-deficient cells expressing less acid SMase and producing less ceramide in response to *P. aeruginosa* infection. A similar defect in acid SMase activity in response to *P. aeruginosa* was observed in CFTR knockout mice.²⁸

There are several mouse models with different *Cftr* mutations which display varying levels of CFTR function,²⁷ and, much like experiments with human airway epithelial cell lines, disparate results regarding changes in ceramide levels. One variable is whether gut *Cftr*-deficiency in CFTR-null mice is corrected by replacement with FABP-driven (intestinal fatty acid binding protein-driven) human CFTR. The impact of gut correction is that these mice do not require a special cholesterol rich diet and the change in diet affects sphingolipid homeostasis.⁸ Nevertheless, while studies in *Cftr*-deficient mouse models report both increased²⁹ and decreased³⁰ ceramide in mouse airways, normalizing pulmonary ceramide levels decreases pro-inflammatory signals.^{29,30}

Inflammation, oxidative stress, and sphingolipid biosynthesis are interconnected; many inflammatory mediators can alter sphingolipid levels by regulating enzymes involved in ceramide generation. For example, oxidative stress and inflammatory cytokines activate acid SMase.³¹ Exposure to cigarette smoke elevates airway neutral SMase, increasing ceramide generation in mice.³² Likewise, cigarette smoke increases neutral SMase in epithelial and endothelial cells, resulting in increased ceramide and subsequent apoptosis in these cell types.³² Notably, increased neutral SMase has been observed in lung tissue from smokers with emphysema³²; however, cigarette smoke exposure is also known to lead to CFTR dysfunction in the lungs,³³ which may impact ceramide homeostasis as discussed above. The relationship between sphingolipid production and oxidative stress is complex. While reactive oxygen species have been reported to affect sphingolipid homeostasis by affecting both enzymatic activity and cellular levels of enzymes involved in sphingolipid production, published data also indicate that ceramide, sphingosine, and sphingosine-1-phosphate can modulate cellular redox. For example, ceramide activates NADPH oxidase, xanthine oxidase, and enzymes in the mitochondrial respiratory chain.³⁴

Excess ceramide induces oxidative stress and increases apoptosis of airway cells.³⁵ Furthermore, increased ceramide induces epithelial and endothelial permeability, promotes susceptibility to *P. aeruginosa* infection,³¹ and activates the inflammasome,³⁶ likely contributing to oxidative stress, inflammation, and altered sphingolipid homeostasis in the CF airway.³ Recently, using a mouse model of intratracheal NE-induced inflammation,^{10–12} we demonstrated that active NE regulates sphingolipid expression.¹³ In this model, oropharyngeal aspiration of exogenous NE increases total ceramide specifically d_{18:1/22:0}, d_{18:1/24:1}, and d_{18:1/24:0} ceramide moieties in murine bronchoalveolar lavage,¹³ suggesting that neutrophilic inflammation may also contribute to the sphingolipid imbalance observed in patients airways. Inhibiting de novo ceramide synthesis in this model using myriocin also decreases the expression of pro-inflammatory signaling molecules—HMGB1 and KC.¹³ Similar to the intratracheal NE mouse model, we observed a correlation between NE activity and long chain ceramide moieties, d_{18:1/22:0} and d_{18:1/24:0}, in sputum from CF patients, and d_{18:1/24:1} ceramide was increased in sputum from patients hospitalized for CF pulmonary exacerbation, compared to samples from the same donors collected during an outpatient visit.

Limited data are available regarding the sphingolipid content in the lung tissue and sputum of patients with CF. Decreased sphingosine and increased ceramide are evident in the nasal epithelium in CF,²⁶ with the latter also noted in lower airways of patients.⁹ Brodlie et al described an association between neutrophilic inflammation and elevated d_{18:1/16:0}, d_{18:1/18:0}, and d_{18:1/20:0} ceramide in explants of human lung tissue from CF subjects. Quinn et al¹⁴ observed that sphingomyelin d_{18:1/14:0}, d_{18:1/15:0}, d_{18:1/16:1}, and d_{18:1/16:0} as well as two d_{18:1/16:0} glycosphingolipids, tetraglycosylceramide and lactosylceramide, were elevated in CF sputum versus non-CF sputum. In a single subject with CF followed for 4.2 years, ceramide was increased during treatment of pulmonary exacerbation.¹⁵ In our study, sputum sphingolipid levels varied among patients and with clinical status, with d_{18:1/14:0} and d_{18:1/24:1} ceramide and d_{18:1/24:0} monohexosylceramide demonstrating a statistically significant increase in subjects during hospitalization for CF pulmonary exacerbation. Ultimately, this report, in concert with published data relevant to COPD and CF, suggests that inflammation modulates airway sphingolipid homeostasis in patients with CF.

Methicillin-resistant *S. aureus* and *P. aeruginosa* are two of the most common bacterial pathogens that chronically infect CF airways; thus, we examined whether the presence of these bacteria in sputum modified the association between active NE and sphingolipid concentration. While ceramide metabolites are critical components in host responses to various bacterial, viral and fungal infections,^{27,32} in a study that focused on sputum metabolomics and bacteriology, Quinn et al. determined that the levels of sphingolipids, including sphingomyelin, ceramide, and lactosylceramide, while substantially different between CF and non-CF sputum samples, did not correlate with the bacterial genera they observed in the CF sputum.¹³ Our data, however, showed that MRSA modified the association of active NE and d_{18:1/14:0} sphingomyelin, total monohexosylceramide, d_{18:1/14:0} and d_{18:1/16:0} monohexosylceramide, and d_{18:1/24:0} ceramide. The presence of MRSA in patient sputum strengthened the association between active NE and the concentrations of these sphingolipids; a notable finding, considering others have reported that *Cfr*-deficient mice are more susceptible to *S. aureus* infection, and that an imbalance between sphingosine

and ceramide may be responsible.³⁷ In contrast, we observed no modifying effects when sputum cultures were positive for *P. aeruginosa*. This corroborates the data reported by Yu et al,²⁸ in which the presence of *P. aeruginosa* stimulated acid SMase activity and ceramide synthesis in normal airway epithelial cells and wild-type mice, but not in *Cftr*-deficient cells or CFTR knockout mice. CFTR knockout mice are more susceptible to *P. aeruginosa* infection, possibly due to a decrease in sphingosine and increase in ceramide in their airways caused by lower enzymatic activity of acid ceramidase.²²

Besides the association between sputum sphingolipids and bacterial infection, we investigated whether patient lung function (FEV₁ % predicted) modified the association between NE and sphingolipids. FEV₁ is known to be inversely associated with NE in CF.¹⁹ However, most our subjects were adults with severe (FEV₁ < 50% predicted) obstructive lung disease, which is why the differences in lung function between hospitalizations and outpatient clinic visits, while statistically significant, were small. Additionally, the difference in sputum NE activity collected during hospitalizations versus outpatient visits were not statistically significant, possibly because sputum donors had relatively high levels of chronic neutrophilic inflammation; similar findings have been reported by others.²¹ When the data collected from all 30 samples were analyzed using a linear mixed model, we observed that higher FEV₁ % predicted weakened the association between NE and sputum sphingolipid content; that is, the better the lung function, the weaker the association between active NE and d_{18:1/14:0} sphingomyelin and d_{18:1/14:0} monohexosylceramide.

Finally, there are data that gender affects the progression of CF, with female patients having increased morbidity and shorter life expectancies.^{38,39} Thus, we assessed the effect of patient gender on the correlation between active NE and sphingolipids. There was an increased association between two sputum sphingolipids— d_{18:1/14:0} sphingomyelin and d_{18:1/14:0} monohexosylceramide, and NE in female subjects. These were the same two sphingolipid moieties that had stronger associations with active NE with poorer lung function. Sphingolipids in serum and plasma are known to vary between genders; for example, healthy female subjects had increased d_{18:1/18:0}, d_{18:1/22:0}, and d_{18:1/24:0} dihydroceramide moieties, and overall demonstrated a trend towards higher levels of dihydroceramides. The significance of these sex-related influences on sphingolipid and NE associations remains to be determined.

While our analysis of CF sputum implies a relationship between active NE and sphingolipid biosynthesis, our study has several limitations. First, the number of samples assessed in this study were few, with only 15 subjects participating in the study. All of the sputum samples in the VCU Biospecimen Repository were spontaneously expectorated, which limits sample availability; not all patients with CF spontaneously expectorate sputum. Additionally, most of the subjects had severe (FEV₁ < 50% predicted) obstructive lung disease, which was reflected by the low, but still statistically significant, difference in FEV₁ % predicted when patients were hospitalized for CF pulmonary exacerbation versus outpatient, as well as the lack of significant difference in active NE observed in sputum between those two sample groups.

The sphingolipids detected in CF sputum could be present due to shedding of sphingolipid-rich extracellular vesicles (eg, microvesicles, exosomes, ectosomes). If so, the cellular source or sources remain unknown. Brodlie et al⁹ observed increased ceramide in lung tissue, specifically in the lower airways in CF patients, and correlated this difference to increased neutrophil elastase and myeloperoxidase positive staining. While there are published reports describing the shedding of bioactive exosomes⁴⁰ and ectosomes⁴¹ by activated neutrophils, it is equally possible that the sphingolipids, including ceramide, that we detected in sputum are derived from airway epithelial cells. This report describes a relationship between active NE and the sphingolipid moieties present in the sputum of patients with CF. Increased NE was associated with higher levels of a variety of sphingolipids in CF sputum, and there was a statistically significant linear correlation between the concentration of active NE and total sphingomyelin, ceramide, monohexosylceramide, and SIP. This novel observation suggests that there exists an association between neutrophilic inflammation and sphingolipid concentrations in CF lungs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Differences in lung function and sputum concentrations of active NE and sphingolipids in patients with CF. Sputum samples were collected during the course of hospitalization or during an outpatient visit ($n = 15$ per group), and processed as described in Section 2. Lung function, expressed as FEV₁ % predicted, varied between the two groups; there was a statistically significant decrease in lung function during hospitalization for CF pulmonary exacerbation (A). Active NE was measured by a chromogenic microtiter plate assay; there was a wide range of NE activity among patients, but no statistically significant difference

between the two sample groups (B). Sphingolipid content was measured by HPLC-ESI-MS/MS. Sputum concentrations of d_{18:1/14:0} ceramide (C), d_{18:1/24:1} ceramide (D), and d_{18:1/24:0} monohexosylceramide (E) varied with clinical status, with increased concentrations of these sphingolipids found in samples collected during hospitalization. The differences between the two groups were statistically significant (*, $P < 0.05$). Data (mean $\pm$ SEM) were compared by Wilcoxon signed rank tests

FIGURE 2.

A linear correlation between the concentration of active NE and total sphingomyelin (A), total ceramide (B), total monohexosylceramide (C), and sphingosine-1-phosphate (S1P) (D) in CF sputum. Sputum samples were collected ($n = 30$ samples, from 15 donors), and processed as described in Section 2. Total sphingolipid content was measured by HPLC-ESI-MS/MS, and the concentration of active NE in sputum was assessed using an NE activity assay. Solid lines depict the best fit of a linear equation to the data. There was a linear correlation between the concentration of active NE and total sphingomyelin ($r^2 = 0.1977$; $P = 0.0138$), total ceramide ($r^2 = 0.1523$; $P = 0.033$), total monohexosylceramide ($r^2 = 0.2293$; $P = 0.0074$), and S1P ($r^2 = 0.2570$; $P = 0.0042$) in CF sputum (A through D, respectively)

FIGURE 3.

Linear correlations between the concentration of active NE and five sphingomyelin moieties in CF sputum. Sputum samples were collected ($n = 30$ samples, from 15 donors), and processed as described in Section 2. Sphingomyelin content was measured by HPLC-ESI-MS/MS, and the concentration of active NE in sputum was assessed using an NE activity assay. Solid lines depict the best fit of a linear equation to the data. Most likely contributors to the observed increase in total sphingomyelin consisted of $d_{18:1/14:0}$ ($r^2 = 0.3641$; $P = 0.0004$), $d_{18:1/18:1}$ ($r^2 = 0.1555$; $P = 0.031$), $d_{18:1/22:0}$ ($r^2 = 0.2933$; $P = 0.0020$), $d_{18:1/24:1}$ ($r^2 = 0.2933$; $P = 0.0020$), $d_{18:1/24:0}$ ($r^2 = 0.2933$; $P = 0.0020$).

= 0.2945; $P = 0.0019$), and $d_{18:1/24:0}$ ($r^2 = 0.1406$; $P = 0.0412$) sphingomyelin (A through E, respectively), all of which demonstrated a correlation with the concentration of active NE in sputum that was statistically significant ($P < 0.05$)

FIGURE 4.

Linear correlations between the concentration of active NE and two ceramide and two monohexosylceramide moieties in CF sputum. Sputum samples were collected ($n = 30$ samples, from 15 donors), and processed as described in Section 2. Ceramide and monohexosylceramide content was measured by HPLC-ESI-MS/MS. The concentration of active NE in sputum was assessed using an NE activity assay. Solid lines depict the best fit of a linear equation to the data. The sphingolipid moieties that likely contributed to the increase in total ceramide were d_{18:1/22:0} ($r^2 = 0.1478$; $P = 0.0359$) (A) and d_{18:1/24:0} ($r^2 = 0.2620$; $P = 0.0038$) ceramide (B). The sphingolipid moieties that likely contributed to the increase in total monohexosylceramide were d_{18:1/16:0} ($r^2 = 0.2725$, $P = 0.0031$) (C) and d_{18:1/24:0} ($r^2 = 0.1641$, $P = 0.0264$) monohexosylceramide (D). The correlation between the

concentration of active NE and these ceramide and monohexosylceramide moieties in sputum was statistically significant ($P < 0.05$)

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Modifying factors that influence the association between NE and sphingolipids in sputum from CF patients

TABLE 1

Modifying factors	Sphingolipid	Change in slope	Standard error	P-value
MRSA positive culture	Sphingomyelin, d _{18:1/14:0}	0.2330	±0.06406	0.0034
	Total monohexosylceramide	0.1825	±0.05539	0.0064
	Monohexosylceramide, d _{18:1/14:0}	13.8162	±3.0586	0.0007
	Monohexosylceramide, d _{18:1/16:0}	0.3669	±0.09921	0.003
	Ceramide, d _{18:1/24:0}	0.5916	±0.2324	0.0257
FEV ₁ % predicted	Sphingomyelin, d _{18:1/14:0}	-0.1644	±0.06836	0.0318
	Monohexosylceramide, d _{18:1/14:0}	-8.8226	±3.8738	0.0403
Female gender	Sphingomyelin, d _{18:1/14:0}	0.2821	±0.07023	0.0015
	Monohexosylceramide, d _{18:1/14:0}	8.1045	±3.6967	0.0472