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# Metabolomic Evaluation of Neutrophilic Airway Inflammation in Cystic Fibrosis

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**BACKGROUND:** Metabolomic evaluation of cystic fibrosis (CF) airway secretions could identify metabolites and metabolic pathways involved in neutrophilic airway inflammation that could serve as biomarkers and therapeutic targets.

**METHODS:** Mass spectrometry (MS)-based metabolomics was performed on a discovery set of BAL fluid samples from 25 children with CF, and targeted MS methods were used to identify and quantify metabolites related to neutrophilic inflammation. A biomarker panel of these metabolites was then compared with neutrophil counts and clinical markers in independent validation sets of lavage from children with CF and adults with COPD compared with control subjects.

**RESULTS:** Of the 7,791 individual peaks detected by positive-mode MS metabolomics discovery profiling, 338 were associated with neutrophilic inflammation. Targeted MS determined that many of these peaks were generated by metabolites from pathways related to the metabolism of purines, polyamines, proteins, and nicotinamide. Analysis of the independent validation sets verified that, in subjects with CF or COPD, several metabolites, particularly those from purine metabolism and protein catabolism pathways, were strongly correlated with neutrophil counts and were related to clinical markers, including airway infection and lung function.

**CONCLUSIONS:** MS metabolomics identified multiple metabolic pathways associated with neutrophilic airway inflammation. These findings provide insight into disease pathophysiology and can serve as the basis for developing disease biomarkers and therapeutic interventions for airways diseases.

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**ABBREVIATIONS:** AMP = adenosine monophosphate; BALF = BAL fluid; CF = cystic fibrosis; EBC = exhaled breath condensate; MS = mass spectrometry

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Chronic neutrophilic airway inflammation is a hallmark of cystic fibrosis (CF) and contributes significantly to morbidity and mortality.[1] This inflammation can alter cellular metabolism, including extracellular metabolic pathways active within the airway lumen that generate biologically active molecules capable of initiating and modulating inflammatory responses.[2-4] Metabolomics studies, which seek to characterize the full range of metabolites within biologic samples, suggest that CF is associated with significant changes in the pattern and concentrations of metabolites in airway secretions, including elevated concentrations of amino acids and lactate in BAL fluid (BALF)[5] and lipid mediators in sputum.[6] Other studies have demonstrated that a metabolomic signal of CF airways disease can be detected noninvasively in exhaled breath condensate (EBC),[7,8] although the specific metabolites responsible for the signal were not identified. Although these studies can provide new insights into pathophysiologic mechanisms and can identify new therapeutic targets and biomarkers of disease progression,[9,10] studies to date are limited by a lack of independent validation to verify results.[11]

We hypothesized that a comprehensive metabolomic analysis of CF airways samples, obtained from subjects with various levels of inflammation, may provide insight into the pathways associated with inflammation and may identify new biomarkers and therapeutic targets. To this end, discovery mass spectrometry (MS) metabolomics was performed in BALF from 25 children with CF, and a biomarker panel of relevant metabolites was developed. The relationships between these metabolites and inflammation, infection, and lung function were then tested in independent validation sets of BALF from children with CF, as well as from adults with COPD and relevant control subjects.

## Materials and Methods

### *Subjects and Samples*

BALF from children with CF was collected during clinically indicated bronchoscopy via standardized protocols[4] and was centrifuged at 11,000 × *g* for 5 min, and the supernatant was stored at −80°C. Fifty pediatric subjects were included: 25 for discovery and 25 for validation (Table 1). BALF from 10 subjects with COPD, 10 healthy smokers, and 10 healthy nonsmokers was obtained using a single lavage of 50 mL normal saline into the right middle lobe. Samples were placed on ice shortly after collection, centrifuged at 11,000 × *g* for 5 min at 4°C to remove cellular material, and then frozen at −80°C until analysis. Both studies were approved by the University of North Carolina institutional review board (IRB Nos. 11-0828 and 05-2876). Clinical data were abstracted from medical and research records.

Metabolomic profiling was performed by the David H. Murdock Research Institute.[12] Briefly, 500-μL aliquots BALF were lyophilized and reconstituted in 100 μL 0.1% formic acid/50% acetonitrile, filtered through 0.2-μm polytetrafluoroethylene, and analyzed on a UPLC-QtofMS system (ACQUITY UPLC-SYNAPT HDMS, Waters Corporation) operated in electrospray ionization positive mode. Chromatography used an ACQUITY UPLC BEH C18 1.7-μM VanGuard precolumn (2.1 × 5 mm) and an ACQUITY UPLC BEH C18 1.7-μM analytical column (2.1 × 100 mm) with gradients from 1% to 100% acetonitrile in 0.1% formic acid over 13 min. Data files were processed using MarkerLynx XS (Waters Corp), with peak detection and signal alignment as described.[12] Analysis occurred in two batches of 10 and 15 samples, both of which included a range of neutrophilic inflammation.

Targeted MS was performed using a Quantum-Ultra triple quadrupole mass spectrometer (Thermo Finnigan LLC) in the multiple reaction monitoring mode. Chromatographic conditions similar to those of the metabolomics (UPLC BEH C18 column, acetonitrile/formic acid gradients) and previous work (UPLC T3 HSS C18 column, methanol/formic acid gradients[13]) were explored, with the T3 column judged superior and used for validation. Validation BALF samples were spiked with isotopically labeled internal standard[14] and were filtered through a 10-kDa-size selection filter (EMD Millipore). Biomarker signals were defined as ratios to the internal standard with the closest column run time.

### *Statistical Analysis*

Discovery metabolomic data were analyzed using robust principal component analysis (Y. H. Z. and J. S. Marron, PhD, unpublished data, 2014). Relationships to inflammation were tested using analysis within each of the two batches, with evidence summarized using Fisher combined *P* value. The false-discovery rate *q* value was used to correct for multiple comparisons. For validation, data not normally distributed (by D'Agostino and Pearson omnibus normality test) were log transformed and analyzed using parametric methods, including Student *t* tests, analysis of variance, and linear regression. Spearman correlations were used for relationships between percent neutrophils to metabolite to urea ratios. Categorical comparisons for demographic balance in discovery vs validation samples were made using the Fisher exact test. Statistical analyses were performed using GraphPad Prism v 5.0 (GraphPad Software, Inc) and SAFExpress software (Safexpress Pvt Ltd).[15]

## Results

### *Subjects and Samples*

Two sets of BALF were obtained from children with CF: a discovery set of 25 samples and a validation set of 25 samples. The sets did not differ in terms of age, sex, or range of inflammation (Table 1), and there was no overlap in subjects. Bacterial or fungal pathogens were recovered in 20 discovery set and 21 validation set samples, with *Staphylococcus*, *Pseudomonas*, and *Haemophilus* species the most common. A second validation set was obtained from adults with COPD, healthy nonsmokers, and healthy smokers (n = 10 per group) (Table 2). Neutrophil counts and percent neutrophils in BALF were higher in those with COPD than in healthy control subjects, but were lower than in patients with CF. All subjects were fasting at the time of the procedure.