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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.

TABLE 1] Demographics of Subjects With CF

Characteristic	Discovery Set	Validation Set	*P* Value for Difference
No.	25	25	...
Age, y	9.6 ± 5.8 (1-21.3)	10.4 ± 3.9 (5-17.7)	.592
Male, %	48	52	.786
Cell count, $\times 10^6$ cells/mL	3.7 ± 3.6 (0.2-14.0)	4.0 ± 4.8 (1.8-18.0)	.868
PMNs, %	61.0 ± 23.7 (4-91.5)	64.2 ± 22.8 (5-89)	.630
PMN count, $\times 10^6$ cells/mL	2.9 ± 3.2 (< 0.1-12)	3.1 ± 3.9 (< 0.1-14.8)	.442
With pathogens, %	80	84	1.000

Data are presented as mean ± SD. CF = cystic fibrosis; PMN = polymorphonuclear leukocyte.

MS Metabolomics on Discovery Samples

Positive-mode MS metabolomics[16] revealed 7,791 individual peaks, with each peak defined as a specific mass-to-charge (m/z) ratio and column run time. Total MS signal correlated positively with neutrophil counts ($r = 0.71$, $P < .001$) (Fig 1A). Principal component analysis revealed a clustering of low-inflammation samples (Fig 1B), with a greater dispersion among the high-inflammation samples.

Potential biomarkers of neutrophilic inflammation were identified by both group (samples divided into groups along median neutrophil count) and correlation analyses (linear regression to neutrophil counts). In group analysis, 267 peaks distinguished higher inflammation (neutrophil counts ≥ 2.0 million cells/mL [n = 13]) from lower inflammation (neutrophil counts ≤ 1.5 million cells/mL [n = 12]) groups (false discovery rate $q < 0.05$) (e-Table 1). For correlation analysis, 140 peaks correlated with neutrophil counts (false discovery rate $q < 0.15$). Sixty-nine peaks overlapped. This resulted in a total of 338 unique peaks identified as potential biomarkers. Several m/z ratios were present in multiple peaks, and 317 unique m/z ratios were represented.

Identification of Potential Biomarkers

Selected ion monitoring was utilized to determine which m/z ratios associated with putative biomarkers generated peaks that were larger in higher- vs lower-inflammation samples. Full scans of each peak were then utilized to determine the fragmentation pattern of the metabolite generating that peak. Metabolite identification was made by comparison with published literature[17] and online resources such as the Human Metabolite Database (www.hmdb.ca) and was confirmed by comparison with appropriate standards. These analyses suggested that the putative biomarkers included the following metabolic pathways (e-Appendix 1).

Purine Metabolism: The putative biomarker peak at m/z 137.1 was identified as hypoxanthine, a metabolic product of adenyl purines identified previously as neutrophilic inflammation biomarkers.[4] In addition, several biomarker peaks observed at m/z 136.1 were consistent with adenine, the primary fragmentation ion of adenyl purines,[13] which may have been generated by in-source fragmentation of adenyl purines.

Polyamine Metabolism: Potential biomarker peaks at m/z 188.1, 146.1, and 89.1 were identified as

TABLE 2] Demographics of Subjects With COPD

Characteristic	Healthy Nonsmokers	Healthy Smokers	Subjects With COPD	*P* Value
No.	10	10	10	...
Age, y	27.6 ± 7.6	30.5 ± 10.0	53.3 ± 9.7	< .01
Male, %	75	67	58	NS
Current smoker,[a] %	0	100	50	ND
FEV_1, % predicted	97.3 ± 10.1	97 ± 11.2	55.7 ± 21.7	< .01
Cell count, $\times 10^6$ cells/mL	2.2 ± 1.2	2.5 ± 1.1	4.3 ± 6.7	.42
PMNs, %	4.1 ± 2.1	6.6 ± 5.4	42.5 ± 33.7	< .01
PMN count, $\times 10^6$ cells/mL	0.09 ± 0.03	0.14 ± 0.09	0.66 ± 0.64	< .01

Data are presented as mean ± SD. ND = not determined; NS = not significant. See Table 1 legend for expansion of other abbreviations.
[a]All subjects with COPD had a history of smoking.

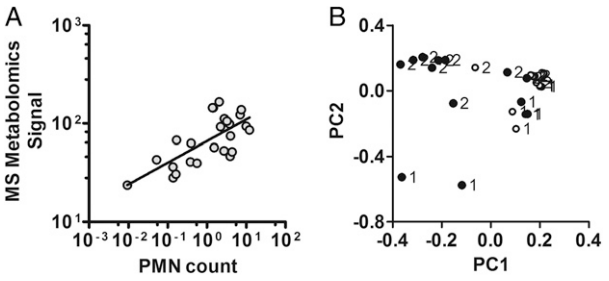

Figure 1 – *MS metabolomics. A, Relationship between neutrophil (PMN) count and total MS signal during discovery metabolomics. Signal for each peak was normalized such that the lowest signal in the dataset had a value of 1. A strong correlation (r = 0.70, P < .001) was observed between neutrophil counts and total MS metabolomics signal. B, Principal component analysis of metabolomic data. Low inflammation samples (○) generally clustered together away from high inflammation samples (●), but with some overlap. MS = mass spectrometry; PC = principal component; PMN = polymorphonuclear leukocyte.*

N-acetyl-spermidine, spermidine, and putrescine, all part of the polyamine synthesis pathway. Furthermore, free adenine can be generated during polyamine synthesis,[18] which may account for one of the previously noted peaks at *m*/*z* 136.1.

Protein Catabolism: Several biomarker peaks had *m*/*z* ratios consistent with that of amino acids; these included proline (*m*/*z* 116.1), valine (*m*/*z* 118.1), threonine (*m*/*z* 120.1), leucine/isoleucine (*m*/*z* 132.1), glutamine (*m*/*z* 147.1), and phenylalanine (*m*/*z* 166.1). Analysis of the internal library suggested that *m*/*z* 188.1 represented an in-source fragmentation product of tryptophan. Free amino acids can be generated by protease activity in CF airways,[19] suggesting that other peptide fragments may also be represented. The biomarker peak at *m*/*z* 229.1 fragmented into major daughter ions at *m*/*z* 116.1 and 132.1, suggestive of a leucine-proline dipeptide, and the peak coeluted with a leucine-proline standard. Other putative biomarkers consistent with dipeptides included those at *m*/*z* 173.1 (glycine-proline), 260.1 (lysine-leucine), 267.1, (threonine-phenylalanine), and 279.1 (leucine-phenylalanine).

Nicotinamide Metabolism: The putative biomarker at *m*/*z* 123.1 was identified as nicotinamide based on an internal library, and its fragmentation pattern (major daughter ion at *m*/*z* 95.1) and chromotographic elution time were identical to those of authentic nicotinamide.

Validation of Potential Biomarkers

Based on these findings, an MS biomarker panel of 30 metabolites was created by modifying a previous MS method[20] to include the putative biomarkers and other metabolites within related metabolic pathways. Correlations with neutrophil counts and clinical measures were then assessed in an independent validation set of BALF samples from 25 children with CF.

Purine Metabolism: The purine nucleotide adenosine monophosphate (AMP) was positively correlated with neutrophilic counts, and adenosine exhibited a trend toward negative correlation (Table 3), both similar to previous observations.[4] Downstream purine metabolites, including inosine, hypoxanthine, and xanthine, were strongly and positively correlated with neutrophilic counts, with hypoxanthine and xanthine exhibiting about three orders of magnitude concentration differences between the lowest and highest inflammation samples.

Polyamine Metabolism: The polyamines putrescine, spermidine, and spermine, as well as S-adenosylmethionine and adenine, positively correlated with neutrophilic inflammation (Table 3). The spermidine to spermine ratio, reportedly reduced in CF exacerbation,[21] exhibited a trend toward negative correlation with neutrophil counts ($r = -0.37$, $P = .07$). Of note, the peak associated with adenine eluted at a run time (about 4.2 min) substantially different from that of authentic adenine standard (about 2.5 min) and could not be confirmed.

Protein Catabolism: Strong correlations with neutrophil counts were observed for multiple amino acids (Table 3), with MS signals varying by more than three orders of magnitude. Strong correlations were observed for multiple dipeptides, consistent with formation during protein catabolism.[19] As a further control, previously described methods[22] were used to demonstrate that the proline-glycine-proline proteolytic fragment of fibronectin, known to correlate with inflammation, also correlated with neutrophil counts ($r = 0.84$, $P < .001$).

Nicotinamide: Nicotinamide and two related metabolites, nicotinamide adenine diphosphate and nicotinamide mononucleotide, were positively, albeit modestly, correlated with neutrophil counts.

Controlling for Dilution

Urea, commonly used as a BALF dilution marker, was included in the MS biomarker panel and correlated with neutrophil counts ($r = 0.73$, $P < .001$). Because this suggested that inflammation may have impacted the dilution of airway secretions in BALF, we performed a secondary analysis of each putative biomarker as a ratio to urea relative to percent neutrophils as a dilution-independent inflammatory marker (e-Table 2). Hypoxanthine, xanthine, and most amino acids and dipeptides remained correlated with neutrophils as ratios to urea,

TABLE 3] Correlations Between Metabolites Identified by MS Metabolomics and Neutrophils in Validation Dataset

Metabolic Pathway	Metabolite	No. Detected	Correlation	
			r	*P* Value
Purine metabolism	AMP	25	0.47	.019
	Adenosine	25	−0.32	.112
	Inosine	25	0.72[a]	<.0001[a]
	Hypoxanthine	22	0.84[a]	<.0001[a]
	Xanthine	24	0.84[a]	<.0001[a]
	Uric acid	24	0.63[a]	.001[a]
Polyamine metabolism	Spermine	25	0.67[a]	<.0001[a]
	Putrescine	25	0.54	.045
	Spermidine	25	0.57	.003
	N-Ac-spermidine	25	0.78[a]	<.0001[a]
	S-adenosyl-methionine	16	0.62	.002
	Adenine-like	23	0.64	.002
Protein catabolism	Proline	25	0.82[a]	<.0001[a]
	Valine	22	0.82[a]	<.0001[a]
	Leucine	25	0.86[a]	<.0001[a]
	Glutamine	25	0.83[a]	<.0001[a]
	Phenylalanine	25	0.87[a]	<.0001[a]
	Tryptophan	25	0.86[a]	<.0001[a]
	Threonine	25	0.83[a]	<.0001[a]
	Alanine	21	0.81[a]	<.0001[a]
	Tyrosine	25	0.87[a]	<.0001[a]
	Leu-Pro	24	0.87[a]	<.0001[a]
	Lys-Leu	22	0.79[a]	<.0001[a]
	Gly-Pro	21	0.75[a]	<.0001[a]
	Thr-Phe	25	0.69[a]	.0001[a]
	Leu-Phe	23	0.75[a]	<.0001[a]
	Pro-Gly-Pro	24	0.84[a]	<.0001[a]
Nicotinamide metabolism	Nicotinamide	25	0.62[a]	.001[a]
	NAD	25	0.46	.021
	NMN	23	0.62	.009

Relationships to neutrophil counts were assessed using Pearson correlations after log transformation. *P* values for correlations are shown.
AMP = adenosine monophosphate; Gly-Pro = glycine-proline; Leu-Phe = leucine-phenylalanine; Leu-Pro = leucine-proline; Lys-Leu = lysine-leucine; MS = mass spectrometry; NAD = nicotinamide adenine diphosphate; NMN = nicotinamide mononucleotide; Pro-Gly-Pro = proline-glycine-proline; Thr-Phe = threonine-phenylalanine.
[a]Significant after Bonferroni multiple-test correction.

but polyamines and nicotinamide were not. However, these results should be interpreted with caution given the limitations of urea as a BALF dilution marker,[23] particularly because urea is generated during polyamine synthesis.[21]

Impact of Infection

Neutrophilic inflammation correlates with the severity of infection,[24] suggesting that airway pathogens may contribute to the metabolomic signal. In fact, biomarkers from each pathway were elevated in samples with bacterial pathogens (n = 15) relative to those with no pathogens (n = 5) (Fig 2). However, biomarker concentrations did not differ between samples that cultured exclusively gram-positive cocci (*Staphylococcus aureus* [n = 7]) vs only gram-negative rods (*Pseudomonas*, *Burkholderia*, or *Stenotrophomonas* species [n = 5]). Correlations between biomarker metabolites and bacterial

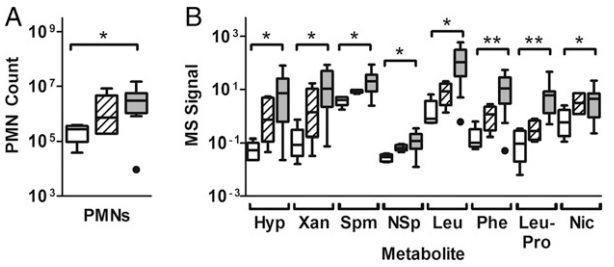

Figure 2 – *Biomarkers and infection. A, Differences in BAL fluid (BALF) neutrophil counts were observed among samples with no pathogens on culture (white, n = 5), fungal pathogens only (hatched, n = 5), or bacterial pathogens (gray, n = 15). *P < .05 by analysis of variance (ANOVA), Tukey posttest indicating differences between no pathogen and bacterial pathogen groups. B, Multiple metabolomics biomarkers also differentiated among BALF culture status, including some metabolites (**) that differentiated samples with bacterial pathogens from both no pathogen and fungal pathogen-only groups (Tukey posttest following ANOVA). Hyp = hypoxanthine; Leu = leucine; Leu-Pro = leucine-proline dipeptide; Nic = nicotinamide; NSp = N-acetyl spermidine; Phe = phenylalanine; Spm = spermine; Xan = xanthine. See Figure 1 legend for expansion of other abbreviations.*

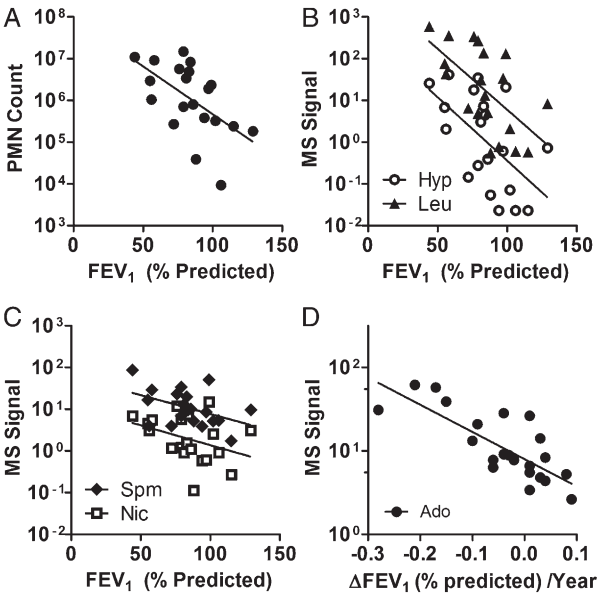

Figure 3 – *BALF biomarkers and lung function. A, Percent predicted FEV_1 was significantly negatively correlated with BALF neutrophil (PMN) counts (r = −0.57, P < .01). B, Similar correlations were observed for BALF purines (Hyp, r = −0.56; Xan, r = −0.56; P < .01) and protein catabolism metabolites (Leu, r = −0.61; Phe, r = −0.59; Leu-Pro, r = −0.59; P < .01). C, Correlations with percent predicted FEV_1 were observed for polyamines (Spm, r = −0.44, P < .05) with a trend for Nic (r = −0.36, P < .11). D, Ado, but no other metabolite, was negatively correlated with changes in predicted FEV_1 over time (r = −0.71, P < .001). Ado = adenosine. See Figure 1 and 2 legends for expansion of other abbreviations.*

counts (organisms/mL) were observed, although they were less robust than correlations with neutrophil counts, and most metabolites remained positively correlated with neutrophil counts even among samples that did not grow bacterial pathogens (Table 4).

Relationship to Clinical Markers

We observed a significant negative correlation between BALF neutrophil counts and percent predicted FEV_1 in our validation set ($r = -0.58$, $P < .01$) (Fig 3A), similar to previous findings.[3] Many biomarker metabolites, including hypoxanthine, xanthine, and several amino acids and dipeptides, were also negatively correlated with percent predicted FEV_1 (Fig 3B). Less robust correlations for polyamines and a trend toward correlation for nicotinamide were also observed (Fig 3C). Biomarker metabolite concentrations were not predictive of changes in FEV_1 following sample collection (average 3.0 ± 0.7 years of data), with the exception of adenosine, which demonstrated a significant negative correlation (Fig 3D). No metabolites were significantly correlated with BMI percentile as a nutritional marker.

TABLE 4] Correlations Between Representative Metabolites and Airway Infection

	Bacterial Counts (n = 15)		PMNs, No Bacteria (n = 10)	
Biomarker	*r*	*P* Value	*r*	*P* Value
Neutrophil counts	0.58	< .05	…	…
Hypoxanthine	0.55	< .05	0.76	< .02
Xanthine	0.60	< .02	0.81	< .01
Spermine	0.38	.16	0.41	.24
N-Ac-Spermidine	0.22	.45	0.62	.06
Leucine	0.55	< .05	0.72	< .05
Phenylalanine	0.59	< .05	0.75	< .05
Leu-Pro	0.31	.28	0.83	< .01
Nicotinamide	0.36	.19	0.79	< .01

See Table 1 and 3 legends for expansion of abbreviations.

Biomarker Panel in COPD

Representative biomarker metabolites were elevated in subjects with COPD relative to healthy nonsmokers, and a subset differentiated those with COPD from healthy smokers (Fig 4A). All metabolites were significantly correlated with BALF neutrophil counts ($r = 0.42$-0.62, $P < .05$ for all), and negative correlations with percent predicted FEV_1 were observed for hypoxanthine ($r = -0.57$), xanthine ($r = -0.39$), leucine ($r = -0.44$), phenylalanine ($r = -0.48$), and nicotinamide ($r = -0.50$) (Fig 4B), with a trend for spermine ($r = -0.32$, $P = .053$).

Discussion

Our data demonstrate that the inflamed CF airway is a metabolically rich environment. Although the airway

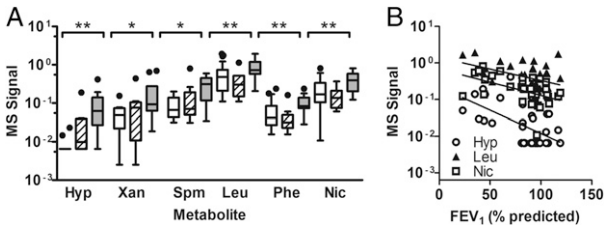

Figure 4 – *Biomarker metabolites in COPD. A, Biomarker metabolites were measured in BALF from healthy nonsmokers (n = 10, white bars), healthy smokers (n = 10, hatched bars), and patients with COPD (n = 10, gray bars). Biomarker metabolites differentiated COPD samples from those of healthy nonsmokers (*) or from both healthy nonsmokers and healthy smokers (**); ANOVA with Tukey posttest. B, Biomarkers Hyp (r = −0.57, P < .001), Leu (r = −0.44, P < .01), and Nic (r = −0.50, P < .01) and others (not shown) were significantly correlated with percent predicted FEV_1. See Figure 1 and 2 legends for expansion of abbreviations.*

metabolome reflects a complex interaction among airway epithelia, inflammatory cells, and microbial pathogens, our data suggest that the metabolites identified in this study primarily reflect neutrophilic inflammation. Correlations were strongest with neutrophil counts, and the purine metabolites hypoxanthine and xanthine, as well as several amino acids, appeared functionally equivalent to neutrophil counts as biomarkers. Measures of these metabolites using simple, commercially available assays could serve as an alternative means of assessing neutrophilic inflammation when standard cell counts are not available, although further investigation is needed to demonstrate the validity and reliability relative to established measures. These biomarkers may be broadly applicable, because they functioned similarly in subjects with CF and COPD.

Our metabolomics studies also identified novel pathways involved in disease pathogenesis. For example, we demonstrated that several adenosine metabolites strongly correlated with airway neutrophils, suggesting that the inflamed CF airway exhibits a robust extracellular metabolism of adenosine that may play a significant role in modulating purinergic signaling responses to inflammation.[25] Accumulation of hypoxanthine in inflamed CF airways is consistent with findings from asthma,[26] bronchopulmonary dysplasia,[27] and ARDS,[28] and may contribute to oxidative stress, because its metabolism to xanthine and uric acid generates oxygen superoxides.[27,28] Thus, existing pharmacologic agents that block hypoxanthine formation[29] may be considered as therapeutic candidates in CF and other airways diseases.

The correlations of airway neutrophils with amino acids and dipeptides are consistent with the known roles of extracellular airway proteases, including those derived from neutrophils, in CF airways disease.[30] Although elevated amino acid concentrations in CF airway secretions has been described previously,[5,31,32] the strong relationships between the markers of protein catabolism and the inflammatory, infection, and clinical measures observed here suggest that these metabolites could serve as biomarkers of in vivo protease activity in trials of protease inhibitors.

Elevated polyamine concentrations have been reported in CF sputum[21] and may increase mucin secretion[33] and aggregation.[34] Interestingly, genetic studies suggest that activity of the methionine salvage pathway, which plays a critical role in polyamine synthesis, is increased in patients with CF who have more severe lung disease.[35] These findings suggest that polyamines may be both a biomarker and a therapeutic target for CF airways disease and COPD.

Nicotinamide is known to have antiinflammatory properties, but an association between nicotinamide and airways disease has not been described previously. Free nicotinamide can be generated by extracellular ADP-ribosyl-transferases[36] that modulate crosstalk between epithelia and neutrophils in inflamed airways.[37] This pathway may be responsible for our findings, although further study is needed.

The metabolites identified in this study could also be used as noninvasive biomarkers of airways disease. MS methods similar to those used in this study have been used to assess EBC as a noninvasive airway sample.[14,20,38] However, although biomarkers such as AMP function similarly in BALF and EBC,[4,38] the relationships between inflammation and adenosine and phenylalanine in the current study differ from previous observations in EBC.[14,20,38] Thus, EBC and BALF may differ in the metabolites that function optimally as biomarkers, perhaps reflecting different sites of origin of the airway secretions,[39,40] and further study will be needed to identify the most effective metabolomic biomarkers for noninvasive detection.

Although we took steps to minimize enzymatic activity after sample collection, we cannot rule out the possibility that extracellular metabolism during sample collection or processing influenced our results. Similarly, although subjects were fasting at the time of sample collection, it is possible that dietary variations altered metabolite concentrations. However, given the strength of our findings, we do not believe that either of these potential confounders would significantly alter out conclusions.

Conclusions

In summary, we used MS metabolomics to identify the metabolites and metabolic pathways associated with neutrophilic airway inflammation in CF and validated these relationships in independent data sets from subjects with CF and COPD. These metabolites can serve as biomarkers of inflammation as well as therapeutic targets.

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