Metabolomic biomarkers predictive of early structural lung disease in cystic fibrosis

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ABSTRACT Neutrophilic airway inflammation plays a role in early structural lung disease in cystic fibrosis, but the mechanisms underlying this pathway are incompletely understood.

Metabolites associated with neutrophilic inflammation were identified by discovery metabolomics on bronchoalveolar lavage fluid supernatant from 20 preschool children (2.9±1.3 years) with cystic fibrosis. Targeted mass-spectrometric detection of relevant metabolites was then applied to 34 children (3.5±1.5 years) enrolled in the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) who underwent chest computed tomography and bronchoalveolar lavage from two separate lobes during 42 visits. Relationships between metabolites and localised structural lung disease were assessed using multivariate analyses.

Discovery metabolomics identified 93 metabolites associated with neutrophilic inflammation, including pathways involved in metabolism of adenyl purines, amino acids and small peptides, cellular energy and lipids. In targeted mass spectrometry, products of adenosine metabolism, protein catabolism and oxidative stress were associated with structural lung disease and predicted future bronchiectasis, and activities of enzymes associated with adenosine metabolism were elevated in the samples with early disease.

Metabolomics analyses revealed metabolites and pathways altered with neutrophilic inflammation and destructive lung disease. These pathways can serve as biomarkers and potential therapeutic targets for early cystic fibrosis lung disease.

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Introduction

Although lungs from children with cystic fibrosis appear anatomically normal at birth [1], progressive airways disease can begin very early in life [2, 3]. Markers associated with neutrophilic inflammation, including IL-8 and neutrophil elastase, are correlated with the extent and progression of lung disease as quantified by computed tomography [4], and the presence of detectable neutrophil elastase in airways of infants is predictive of later bronchiectasis [5]. These findings strongly suggest that neutrophilic inflammation plays a role in early disease; however, the pathophysiological events involved in disease initiation and progression remain ill-defined. Identifying these events is complicated by variable and heterogeneous nature of early cystic fibrosis lung disease that typically involves only a relatively small fraction of the lung [6].

The airway processes involved in early cystic fibrosis lung disease alter cell trafficking and metabolism, including airway surface metabolic pathways and accumulation of inflammatory cells within airway lumens that generate biologically active molecules [7–9]. This scenario suggests that metabolomics approaches, which attempt to characterise the full range of metabolites within biological samples, can elucidate the pathophysiological changes related to inflammation and structural disease. Indeed, previous metabolomics studies of airway secretions have demonstrated that neutrophilic airway inflammation in cystic fibrosis patients with established disease is characterised by increased concentrations of metabolites from several pathways, including those involved in cellular energy [10], protein catabolism [10, 11], adenyl purine metabolism [11] and lipid signalling molecules [12]. However, the relevance of these pathways to early disease has not been evaluated.

We hypothesised that metabolomics studies on airway secretions from young children with cystic fibrosis would identify the metabolic pathways associated with neutrophilic airway inflammation, and that these pathways would be predictive of early structural lung disease. To test these hypotheses, we performed mass spectrometric metabolomics on supernatants of bronchoalveolar lavage fluid (BALF) obtained during clinically indicated bronchoscopy in preschool children with cystic fibrosis to identify pathways associated with neutrophilic inflammation. We then studied relationships between identified metabolic pathways and early localised structural disease in a separate cohort of preschool children enrolled in the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) who underwent chest computed tomography and lavage at a time of clinical stability. To address disease heterogeneity, bronchoalveolar lavage (BAL) samples were obtained from two separate lobes and analysed using statistical modelling to examine relationships between BALF metabolite concentrations and both current and future lobe-specific computed tomography scores.

Methods

Subjects and samples

For discovery metabolomics, BALF from 20 preschool children with cystic fibrosis was collected during clinically indicated bronchoscopy at the University of North Carolina at Chapel Hill (UNC–CH) *via* standardised protocols [9] using one–three 10 mL·kg^{-1} aliquots of sterile saline lavaged into the most visually affected lobe on each side. Return from both sides was combined and averaged 44±15% of lavaged volume. BALF aliquots were centrifuged at 11 000×*g* for 5 min, and the supernatant stored at −80°C. Clinical data were abstracted from medical and research records. All children were fasted for >6 h at the time of collection.

For comparison with structural lung disease, chest computed tomography and BAL were performed on 34 children enrolled in AREST CF as described [2, 6] during 42 study visits (eight subjects were studied during two annual study visits). For BAL, the right middle lobe and the lingula were lavaged and the first aliquot from each side was processed separately, yielding two BAL aliquots per subject visit. BAL samples were centrifuged to remove cellular debris and supernatants were frozen at −80°C and shipped to UNC–CH on dry ice.

From the chest computed tomography scan, each lung lobe was assessed for lobe-specific bronchial wall thickening (BWT) and bronchiectasis using the modified cystic fibrosis–computed tomography (CF–CT) scoring system [2, 13] as well as PRAGMA–CF (Perth–Rotterdam Annotated Grid Morphometric Analysis for Cystic Fibrosis) [14] to give continuous, lobe-specific structural lung disease scores. 29 computed tomography images obtained from individual children 1 year following the BALF samples were also assessed to determine the predictive value of identified biomarkers.

Studies were approved by the UNC institutional review board (numbers 07-0787 and 12-1538) and the Princess Margaret Hospital for Children, Perth and Royal Children's Hospital, Melbourne, ethics committees (registration number 1762/EP).

Mass spectrometry metabolomics

Metabolomic profiling was performed by Metabolon, Inc. (Durham, NC, USA) as previously described [15] using three independent platforms (ultra-high-performance liquid chromatography/tandem mass spectrometry

for acidic and basic metabolites as well as gas chromatography/mass spectrometry). Metabolites were identified by automated comparison of ion features to a reference library. Values below limits of detection were imputed from the minimum detectable value. The average time between sample collection and analysis was 290 days, with a range of 110–483 days.

Targeted mass spectrometry

Targeted mass spectrometry utilised a Quantum–Ultra triple quadrupole mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) with chromatographic conditions similar to those previously described (UPLC T3 HSS C18 column, methanol/formic acid gradients [16]). BALF samples were spiked with isotopically labelled internal standard [17] and filtered through a 10 kDa size selection filter (EMD Millipore, Billerica, MA, USA). Biomarker signals were defined as ratios to the internal standard with the closest column run time. The average time between sample collection and analysis was 268 days, with a range of 39–473 days.

Adenosine metabolism

Adenosine metabolism in BALF was assessed by measuring hypoxanthine generated after incubating 5 µL BALF supernatant with 200 µM adenosine in 50 µL Tris pH 7.5 at 37°C for 1 h. Resulting hypoxanthine was assessed using a reaction mix containing Amplex red, horseradish peroxidase, and xanthine oxidase from the Xanthine Oxidase Fluorometric Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA). The signal was measured on a fluorometric plate reader with excitation at 530 nM and absorbance at 570 nM.

Statistical analysis

Mass spectrometry signals from metabolomic data were analysed using linear regression as well as t-test, with the false-discovery rate q-value used to correct for multiple comparisons. Mass spectrometry data were not normally distributed (by the D'Agostino and Pearson omnibus normality test) and were log transformed prior to analysis. Categorical comparisons for demographic balance in discovery *versus* validation samples were made using Fisher's exact test. CF–CT scores were analysed as binary outcomes of no disease (no BWT or bronchiectasis) *versus* disease (BWT or bronchiectasis present). General estimating equations models were fitted for each metabolite with binomial family, logit link, robust standard errors and were adjusted for sex and batch/lung lobe interactions where appropriate. PRAGMA continuous computed tomography outcomes (% disease and % bronchiectasis) were analysed using hierarchical mixed-effects models with random intercepts for each participant and random intercepts and slopes for each batch (as a function of metabolite), adjusted for sex. Predictive ability of metabolites was investigated by hierarchical mixed effects models described above and by plotting receiver operating characteristic (ROC) curves for presence of bronchiectasis at 12 months computed tomography follow up. Area under the curve estimates and 95% confidence intervals were calculated using ROC regression adjusted for the lung lobe (right middle lobe or lingula) and PRAGMA % bronchiectasis at baseline. Statistical analyses were performed using GraphPad Prism v5.0 (San Diego, CA, USA) and Stata (version 13.0; StataCorp, College Station, TX, USA). Tukey boxplots are used for error bars and outliers.

Results

Discovery metabolomics was performed on BALF supernatants from 20 preschool children with cystic fibrosis undergoing clinically indicated bronchoscopy (table 1). Persistent cough was the indication for most subjects (14 out of 20), with evaluation of new *Pseudomonas* infection (three out of 20) or surveillance in conjunction with another procedure (three out of 20) less common indications. Respiratory pathogens were recovered from culture in 60% of samples, with *Staphylococcus aureus* and *Pseudomonas aeruginosa* most commonly identified (table 1). Samples for metabolomics were chosen to reflect a range of airway inflammation, with six samples having neutrophilic bronchitis (% neutrophils $\geqslant$60%, >50 000 pathogens per mL on culture), nine having no or mild bronchitis (% neutrophils <40%, $\leqslant$10 000 pathogens per mL on culture), and five samples with intermediate values. A total of 152 metabolites were detected in at least one sample. Samples with greater airway neutrophilia had more overall mass spectrometry signal, particularly those with % neutrophils >60% (figure 1).

Metabolites associated with neutrophilic inflammation were defined as those correlated with percent neutrophils at r>0.5 or that had >2-fold increases in signal in samples with bronchitis *versus* those with no or mild bronchitis at a false discovery rate <0.05. This included 93 metabolites that fell into four broad metabolic pathways (table 2 and supplementary table S1).

Adenyl purines and related metabolites

AMP was 4.7-fold elevated in airways with bronchitis (table 2), but adenosine was reduced in bronchitic airways; the only metabolite in discovery metabolomics significantly decreased in the presence of bronchitis. In addition, the adenosine metabolites hypoxanthine and xanthine were 4.8-fold and 15.1-fold

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TABLE 1 Subject demographics

	Discovery set	AREST CF set
Subjects n	20	34
BALF n	20	84
Age years	2.9±1.3 (0.48–4.95)	3.5±1.5 (0.96–5.83)
Male %	55	43
BMI Z-score	0.1±1.1	0.0±1.8
Treatment %		
Hypertonic saline	6	15
Dornase alpha	3	35
Amoxicillin/clavulanate	48	0
Cell count $\times 10^6$ cells·mL^{-1}	2.8±3.7 (0.18–12.94)	0.7±0.3 (0.06–1.67)
PMNs %	48.8±27.2 (2.5–93)	20.2±23.9 (0.67–92)
Pathogens %	60	17
***Staphylococcus* spp. %**	25	7
***Pseudomonas* spp. %**	25	2

Data are presented as mean±SD (range) or mean±SD, unless otherwise stated. AREST CF: Australian Respiratory Early Surveillance Team for Cystic Fibrosis; BALF: bronchoalveolar lavage fluid; BMI: body mass index; PMNs: polymorphonuclear leukocytes.

increased in BALF from bronchitic airways, respectively. The purine-containing compound, nicotinamide adenine dinucleotide (NAD), was also elevated in bronchitis, as were free nicotinamide and kynurenine, a tryptophan metabolite that serves as an intermediate in NAD synthesis.

Amino acids, small peptides and related pathways

A large number of amino acids, dipeptides and tripeptides were highly elevated (>10-fold) in airways with bronchitis, with correlation coefficients to % neutrophils often exceeding 0.7 (table 2). Similarly, bronchitic airways had higher concentrations of several metabolic products of amino acids, including *N*-acetylated derivatives of methionine, serine and lysine. Higher concentrations oxidative products of the antioxidant peptide glutathione, including glutathione disulfide (2.5-fold increased) and cysteine-glutathione disulfide (9.0-fold increased), were also observed.

Arginine-related signalling pathways were also implicated in the metabolomics analysis. Arginine, citrulline and fumarate are involved in generating the signalling molecule nitric oxide [18], and all of these metabolites were elevated in bronchitic samples. Arginine can also be metabolised to ornithine, the initial substrate in synthesis of polyamines [19]. Both ornithine and the polyamine putrescine were elevated in bronchitis, as was free adenine generated primarily within the polyamine synthesis pathway [20].

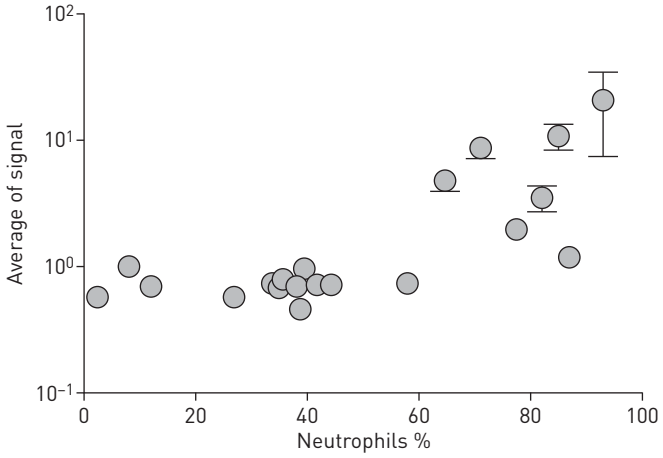

FIGURE 1 Average mass spectrometry metabolomics signal in bronchoalveolar lavage fluid from 20 preschool children with cystic fibrosis plotted relative to % neutrophils on cell counts from these samples. Samples with >60% neutrophils had more overall mass spectrometry signal.

TABLE 2 Selected metabolites related to neutrophilic airway inflammation in discovery metabolomics

Pathway and metabolite	Corrected % PMNs	Ratio[#]	Q-value
Adenyl purine metabolism			
Adenosine 5′-monophosphate	0.60	4.7	0.040
Adenosine	−0.69	0.2	0.002
Hypoxanthine	0.54	4.8	0.002
Xanthine	0.76	15.1	0.000
Urate	0.34	2.4	0.031
Nicotinamide	0.45	3.2	0.006
Nicotinamide adenine dinucleotide	0.63	3.5	0.022
Kynurenine	0.65	2.5	0.016
Amino acids, dipeptides and related metabolites			
Alanine	0.68	36.9	0.000
Phenylalanine	0.74	36.2	0.000
Tyrosine	0.75	44.1	0.000
Leucine	0.37	5.4	0.058
Isoleucine	0.61	6.6	0.002
Methionine	0.60	2.7	0.005
Serine	0.76	20.0	0.000
Lysine	0.75	141.8	0.000
Valine	0.76	43.6	0.000
Aspartylleucine	0.78	29.6	0.000
Aspartylphenylalanine	0.73	15.0	0.000
Glycylleucine	0.73	33.9	0.000
Lysylleucine	0.79	5.4	0.000
N-acetylmethionine	0.45	2.5	0.032
N-acetylserine	0.59	2.5	0.008
N6-acetyllysine	0.76	12.0	0.000
Cysteine-glutathione disulfide	0.69	9.0	0.000
Glutathione, oxidised	0.34	2.5	0.022
Arginine	0.63	29.2	0.000
Citrulline	0.79	24.3	0.000
Fumarate	0.43	2.2	0.009
Ornithine	0.75	71.7	0.000
Putrescine	0.35	6.5	0.061
Adenine	0.40	2.9	0.011
Cellular energy			
Glucose	0.65	9.2	0.002
Citrate	0.66	4.1	0.009
Malate	0.66	2.9	0.004
Lactate	0.71	4.1	0.000
Carnitine	0.43	3.2	0.009
Acetylcarnitine	0.50	3.6	0.003
1,5-anhydroglucitol	0.81	7.0	0.000
Lipids			
Phosphoethanolamine	0.52	4.4	0.003
Cholesterol	0.67	2.1	0.009
Arachidonate (20:4n6)	0.64	9.2	0.003
Myo-inositol	0.43	2.3	0.023
1-stearoyl glycerphosphoethanolamine	0.68	2.5	0.030
2-arachidonoyl glycerphosphoethanolamine	0.65	4.1	0.022
2-docosahexaenoyl glycerphosphoethanolamine	0.66	2.7	0.009
2-oleoyl glycerphosphoethanolamine	0.73	5.4	0.005

[#]: ratio of metabolite concentrations in samples with bronchitis to those with no/mild bronchitis.

Cellular energy metabolism

Several metabolites directly related to energy metabolism were found at higher concentrations in samples from bronchitic airways, including glycolytic and Krebs cycle metabolites (glucose, citrate and malate). Lactate was 4.1-fold increased, suggestive of anaerobic metabolism. Metabolites involved in fatty acid

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oxidation, including carnitine and acylcarnitine, were also elevated. Metabolomics also revealed elevated concentrations of 1,5-anhydroglucitol in bronchitis, a compound that regulates glycaemic control and has anti-inflammatory properties [21].

Lipids

Several lipid metabolites were elevated in the presence of bronchitis, including both common cell membrane lipids (phosphoethanolamine and cholesterol) and those involved in signalling pathways (arachidonate and myo-inositol). Several lysolipids were also elevated in bronchitis.

Metabolomic biomarkers and early structural lung disease

To determine relationships to early structural lung disease, we developed targeted mass spectrometry methods for a subset of 28 metabolomic biomarkers amenable to mass spectrometry detection using previously established methods [11, 16] including amino acids, dipeptides, adenyl purines, nicotinamide, polyamines, glutathione and glutathione disulfide (oxidised glutathione), as well as urea as a potential dilution marker (supplementary table S1). This biomarker panel was then applied to a validation set of samples from 34 preschool children enrolled in AREST CF during 42 study visits (eight longitudinally sampled subjects). In contrast to the subjects in the discovery set, bronchoscopy and chest computed tomography in AREST CF are performed at a time of clinical stability, as evidenced by lower neutrophil counts and fewer recovered pathogens compared with our discovery dataset (table 1). Heterogeneity of early cystic fibrosis lung disease was addressed by obtaining BALF independently from two separate lobes (right middle lobe and lingula) at each study visit and using lobe-specific chest computed tomography scores for analysis.

Metabolomic biomarkers and inflammatory markers

Conventional inflammatory markers including cell counts and IL-8 were measured in samples from the right middle lobe in all subjects (n=42). Analyses revealed significant negative associations (coefficient (95% CI)) between BAL neutrophil counts (% of total cell count) and adenosine (−0.376 (−0.569–−0.184)) and glutathione (−0.285 (−0.419–−0.150)). Significant positive associations were found between neutrophils and inosine (0.220 (0.042–0.399)) and ornithine (0.017 (0.008–0.025)). For IL-8, a significant negative association was found to glutathione (−0.137 (−0.266–−0.007)), with significant positive associations to inosine (0.365 (0.227–0.503)), leucine (0.071 (0.004–0.139)), ornithine (0.012 (0.004–0.020)) and spermidine (0.010 (0.005–0.016)). No significant associations were observed between any metabolomic biomarker and neutrophil elastase, though these analyses were limited by the fact that neutrophil elastase was detected in only five out of 42 samples.

Metabolomic biomarkers and structural lung disease

Statistical modelling was utilised to assess relationships between various metabolites and lobe-specific structural lung disease using both dichotomous variables (presence/absence of disease) and the PRAGMA continuous scoring system. Lobes with structural lung disease (BWT as a marker of early disease or bronchiectasis as a marker of later disease) had lower concentrations of adenosine (OR 0.32; $p<0.001$; figure 2 and table 3) than samples from lobes without BWT or bronchiectasis. Trends towards increases in the downstream adenosine metabolites hypoxanthine (OR 2.32; $p=0.203$) and xanthine (OR 2.30; $p=0.197$) were also observed. Glutathione was significantly reduced in lobes with structural disease (OR 0.40; $p<0.01$), as was oxidised glutathione (OR 0.16; $p<0.01$). Associations between amino acids and structural lung disease were not statistically significant, but the Leu–Pro dipeptide was strongly elevated in lobes with structural lung disease (OR >100; $p<0.001$).

Using lobe-specific PRAGMA (Perth–Rotterdam Annotated Grid Morphometric Analysis) disease scores, we observed negative correlations for both adenosine ($\beta=-0.74$, $p=0.014$) and glutathione ($\beta=-0.82$, $p=0.008$) (table 3). In contrast, positive correlations were observed for the adenosine metabolites hypoxanthine ($\beta=0.93$, $p=0.003$) and xanthine ($\beta=1.46$, $p<0.001$) as well as the amino acids phenylalanine, tyrosine, and the Leu–Pro dipeptide ($\beta=2.14$, 2.13, 2.16 respectively, $p<0.001$ for all). Similar findings were observed for bronchiectasis-specific PRAGMA scores (table 3).

Predictive power of metabolomic biomarkers

Hierarchical mixed effects models and ROCs were utilised to assess the ability of metabolites to predict development of new bronchiectasis at the next annual visit, using data available in 29 children. Metabolites predictive of future bronchiectasis included hypoxanthine ($\beta_1=0.19$ (0.06–0.31); $p=0.003$), phenylalanine ($\beta_1=0.27$ (0.11–0.43)) and Leu–Pro ($\beta_1=0.17$ (0.01–0.33)). Each of these metabolites was a better predictor of bronchiectasis than neutrophil elastase ($\beta_1=0.08$ (0.00–0.16)), a known biomarker of early cystic fibrosis lung disease (figure 3) [5].

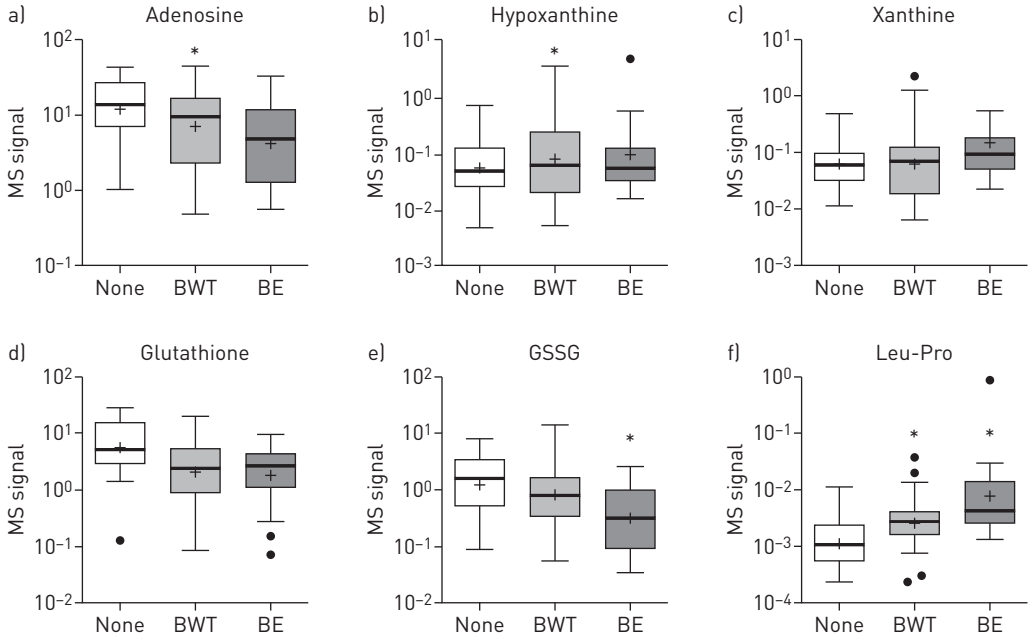

FIGURE 2 Metabolite concentrations for various metabolites in lobes with bronchial wall thickening (BWT), bronchiectasis (BE), or neither (none) by cystic fibrosis–computed tomography scoring system. Circles indicate outliers and + indicates mean. MS: mass spectrometry; GSSG: glutathione disulfide. *: p<0.05 by multivariate analysis.

Early structural lung disease and adenosine metabolism

These analyses suggested an association between early lung disease and activity of the adenosine metabolic pathway, in which adenosine is converted to uric acid through the actions of adenosine deaminase, purine nucleotide phosphorylases, and xanthine oxidase. To assess these activities directly, we measured adenosine metabolism in BALF supernatants. BALF samples from children with significant bronchitis, but not those without bronchitis, metabolised adenosine to hypoxanthine (figure 4), indicating elevated activities of adenosine deaminase and purine phosphorylase. AREST CF samples from lobes with bronchiectasis also had significant adenosine metabolic activity. Xanthine oxidase activity in these samples was not detected (not shown).

Discussion

Using metabolomics, we identified several metabolites and metabolic pathways altered in the presence of neutrophilic airway inflammation in young children with cystic fibrosis, including those involving adenyl purines, amino acids and peptides, cellular energy and lipids. Several of these pathways, including those related to adenosine metabolism, oxidative stress and protein catabolism, were strongly associated with structural lung disease. Although many of these pathways are also altered in older children [10, 11], this study demonstrates that these metabolic changes occur early in the disease process before the onset of permanent structural lung damage. These pathways represent potential therapeutic targets, and the relevant metabolites are biomarkers of patients at risk for developing bronchiectasis.

TABLE 3 Multivariate analysis of metabolite signal and localised structural lung disease

	Disease presence (CF-CT scores)		PRAGMA-CF % disease		PRAGMA-CF % bronchiectasis	
	Adjusted OR (95% CI)	p-value	β (95% CI), R^2	p-value	β (95% CI), R^2	p-value
Adenosine	0.32 (0.18, 0.59)	<0.001	−0.74 (−1.34, −0.15), 0.13	0.014	−0.84 (−1.27, −0.40), 0.18	<0.001
Hypoxanthine	2.32 (0.53, 10.14)	0.203	0.93 (0.32, 1.54), 0.20	0.003	0.76 (0.30, 1.23), 0.19	0.001
Xanthine	2.30 (0.65, 8.20)	0.197	1.46 (0.82, 2.10), 0.32	<0.001	1.2 (0.70, 1.70), 0.31	<0.001
Glutathione	0.40 (0.22, 0.74)	0.003	−0.82 (−1.41, −0.21), 0.14	0.008	−0.55 (−1.10, −0.00), 0.11	0.048
Phenylalanine	3.08 (0.44, 21.71)	0.259	2.14 (1.44, 2.85), 0.44	<0.001	1.65 (1.07, 2.24), 0.38	0.001
Tyrosine	1.60 (0.78, 3.27)	0.203	2.13 (1.43, 2.83), 0.41	<0.001	1.5 (0.83, 2.18), 0.39	0.001
Leu-Pro	2.80×10^{70} (2.01×10^{17}, 3.9×10^{123})	0.009	2.16 (1.61, 2.71), 0.52	<0.001	1.65 (1.25, 2.06), 0.51	<0.001

CF: cystic fibrosis; CT: computed tomography; PRAGMA–CF: Perth–Rotterdam Annotated Grid Morphometric Analysis for Cystic Fibrosis.

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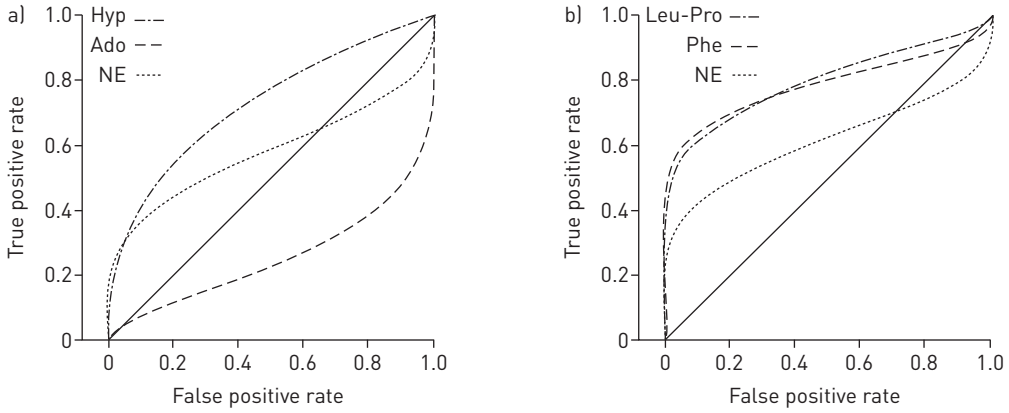

FIGURE 3 Receiver operating characteristic curves demonstrating the sensitivity and specificity for various metabolites relative to neutrophil elastase (NE) in predicting future bronchiectasis. a) Purine metabolites adenosine (Ado) and hypoxanthine (Hyp); and b) protein catabolism metabolites phenylalanine (Phe) and the dipeptide Leu–Pro.

The adenosine metabolic pathway in particular appears altered early and predictive of future disease. Adenosine plays an important and complex role in modulating signalling responses to inflammation [22], with both pro and anti-inflammatory properties, though normal airway adenosine concentrations are thought to be anti-inflammatory [22, 23]. The decreased adenosine and elevated adenosine metabolic activity likely increase airway inflammatory responses and the metabolic products hypoxanthine and xanthine contribute to oxidative stress through metabolism by xanthine oxidase, which generates oxygen superoxides [24, 25].

Increased oxidative stress in early disease is consistent with the observed reduction in glutathione, the primary antioxidant in the airway [26]. Decreased lower airway glutathione concentrations have been observed in preschool children with cystic fibrosis [27], although our study is the first to demonstrate a relationship to structural lung disease. Our ability to detect these relationships likely reflects our use of lobe-specific lavages and computed tomography scores to account for disease heterogeneity. Somewhat surprisingly, the concentrations of oxidised glutathione were also reduced in the AREST CF samples, though we suspect this may reflect an inability to preserve the oxidative state on storage and transport of these samples.

These findings suggest that drugs that affect the adenosine metabolism are potential therapeutic targets in cystic fibrosis. Such drugs could represent "low-hanging fruit," since several relevant pharmaceuticals are approved or in late-stage clinical trials. For example, inhibitors of purine nucleoside phosphorylase that block hypoxanthine formation [28] are in clinical trials for gout. Also, the xanthine oxidase inhibitor febuxostat was recently approved for gout [29] but has also been shown to reduce airway inflammation in an animal model of acute lung injury [30]. Although we did not detect xanthine oxidase activity in BALF supernatant, the high concentrations of xanthine and uric acid (metabolic products of xanthine oxidase) in

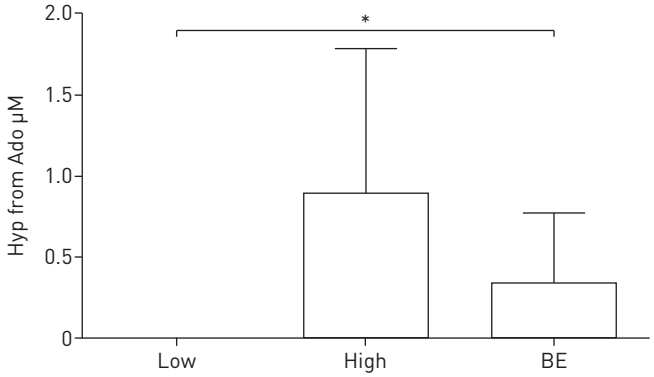

FIGURE 4 Adenosine metabolic activity in bronchoalveolar lavage fluid (BALF) from preschool children with cystic fibrosis was assessed as the ability to generate hypoxanthine (Hyp) from exogenously added adenosine (Ado). Samples with no significant bronchitis (<40% neutrophils) had no significant activity, whereas activity was readily detected in samples with bronchitis (>60% neutrophils). BALF from clinically stable preschoolers with bronchiectasis (BE) on chest computed tomography showed moderate levels of adenosine metabolic activity. n=3–4 per group. *: p<0.01 by Mann–Whitney, with significant post-tests for bronchitis and BE groups relative to no bronchitis.

the airway samples indicate that this enzyme is active *in vivo*, likely restricted to the airway epithelial cell surface. Indeed, our adenosine metabolism studies may underestimate total activity since we could only assess soluble activities and not the contribution from enzymes found on airway surfaces.

The strong associations between amino acids and dipeptides with early disease are consistent with increased activity of proteases such as neutrophil elastase. These findings are supportive of previous studies identifying airway proteases as potential therapeutic targets in early cystic fibrosis lung disease [5]. Notably, the Leu–Pro dipeptide was more readily detected and more predictive than neutrophil elastase, suggesting that it and similar metabolomics biomarkers may be more sensitive indicators of protease activity.

Discovery metabolomics suggested that several arginine-related signalling pathways are upregulated in the presence of airways inflammation. Increased concentrations of ornithine imply greater arginase activity in inflamed airways and are consistent with previously reported increases in cystic fibrosis sputum polyamines [19]. Since urea is another product of arginase activity, this relationship could impact the utility of urea as an airway dilution marker. Increased concentrations of citrulline and fumarate were also observed, suggesting greater flux through the nitric oxide synthesis pathway. However, exhaled nitric oxide is reportedly low in cystic fibrosis [31]. The reasons for this discrepancy are not clear.

In fact, several metabolites identified in our study have potential as biomarkers of early lung disease. Many of these metabolites are detectable by conventional methods and could potentially serve as indicators of at risk children that are more sensitive than the current gold standard of neutrophil elastase [5]. In addition, some of the metabolic signatures observed in BALF are detectable in exhaled breath condensate from young children [32, 33], offering the potential to develop a relatively noninvasive technique to identify children with early structural lung disease.

There were several limitations that could affect our findings. All subjects were fasting at the time of bronchoscopy, and it is possible that metabolic patterns would differ in non-fasting individuals. We also did not assess the impact of treatment beyond an indirect effect of altering airway inflammation. Similarly, we were not able to determine the direct contribution of bacterial pathogens to the metabolomic signal. However, given the complexity of the cystic fibrosis airway microbiome [34], assessing the relative contributions of host *versus* bacterial pathogens poses considerable challenges.

Another limitation is that we utilised % neutrophils to define airway inflammation in our discovery metabolomic analysis. While airway neutrophils are a well-accepted marker of inflammation [8], this approach does have some shortcomings since increases in other cell counts (such as lymphocytes during an acute viral infection) or sampling issues could alter the relationship between % neutrophils and airway inflammation. Similarly, neutrophil counts alone may not perfectly reflect their activity or propensity to cause airway damage. Nevertheless, we did observe associations between many of the metabolites and metabolic pathways identified by discovery metabolomics and other inflammatory markers as well as structural lung disease. These associations raise our confidence that our findings reflect early cystic fibrosis airways disease pathophysiology.

This study focused on metabolites amenable to our established mass spectrometry methods, and we have not yet analysed other metabolites identified by discovery metabolomics such as those involved in cellular energy and lipid metabolism. These metabolites will require different mass spectrometry approaches for detection, but they offer fertile ground for further investigation.

In conclusion, mass spectrometry metabolomics demonstrate that neutrophilic airway inflammation in young children with cystic fibrosis is associated with increased concentrations of metabolites from many pathways, several of which are predictive of current and future early structural lung disease. In particular, alterations in adenosine metabolism and resulting oxidative stress are linked to both bronchitis and structural lung disease and offer opportunities for noninvasive biomarker detection and serve as promising targets for therapeutic intervention.

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