Published in final edited form as:

Pediatr Pulmonol. 2015 September ; 50(9): 869–877. doi:10.1002/ppul.23225.

Metabolomics Analysis Identifies Novel Plasma Biomarkers of Cystic Fibrosis Pulmonary Exacerbation

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Summary

Background—Cystic fibrosis (CF) lung disease is characterized by infection, inflammation, lung function decline, and intermittent pulmonary exacerbations. However, the link between pulmonary exacerbation and lung disease progression remains unclear. Global metabolomic profiling can provide novel mechanistic insight into a disease process in addition to putative biomarkers for future study. Our objective was to investigate how the plasma metabolomic profile changes between CF pulmonary exacerbation and a clinically well state.

Methods—Plasma samples and lung function data were collected from 25 CF patients during hospitalization for a pulmonary exacerbation and during quarterly outpatient clinic visits. In collaboration with Metabolon, Inc., the metabolomic profiles of matched pair plasma samples, one during exacerbation and one at a clinic visit, were analyzed using gas and liquid chromatography coupled with mass spectrometry. Compounds were identified by comparison to a library of standards. Mixed effects models that controlled for nutritional status and lung function were used to test for differences and principal components analysis was performed.

Results—Our population had a median age of 27 years (14–39) and had a median FEV1% predicted of 65% (23–105%). 398 total metabolites were identified and after adjustment for confounders, five metabolites signifying perturbations in nucleotide (hypoxanthine), nucleoside (N4-acetylcytidine), amino acid (N-acetylmethionine), carbohydrate (mannose), and steroid (cortisol) metabolism were identified. Principal components analysis provided good separation between the two clinical phenotypes.

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Conflict of interest: None.

This work has previously been published in abstract form at the North American Cystic Fibrosis Conference, Salt Lake City, UT; October 2013.

Conclusions—Our findings provide putative metabolite biomarkers for future study and allow for hypothesis generation about the pathophysiology of CF pulmonary exacerbation.

Keywords

inflammation; infection; lung function

Introduction

Cystic fibrosis (CF) lung disease begins silently in infancy and is characterized by infection, inflammation, lung function decline, and pulmonary exacerbations.¹ Neutrophilic inflammation in bronchoalveolar lavage fluid (BALF) and pulmonary infection with *Pseudomonas aeruginosa* are major risk factors for more severe CF lung disease in children.^{2–6} Pulmonary exacerbations are associated with a lack of short term recovery and a subsequent longitudinal decline in lung function.^{7–9} Given life expectancy is tightly linked to the degree of airway obstruction [i.e. forced expiratory volume in one second (FEV₁)], patients are closely monitored for known risk factors and pulmonary exacerbations are treated aggressively. However, significant gaps in knowledge remain with disease severity and lung function decline known to vary widely within patients carrying the same mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. There are urgent needs for clinically relevant biomarkers to aid in the earlier identification of a CF pulmonary exacerbation and to define the mechanistic links between inflammation, pulmonary exacerbations and lung function decline.¹⁰

Metabolomics is the global assessment of endogenous metabolites within a biological system, identifying potential biomarkers, and providing novel mechanistic insight into a disease process. In addition to identifying putative biomarkers, metabolomic profiling provides an instantaneous snapshot of the physiology of an organism and can be a direct link to the mechanism of disease.¹¹ Changes in metabolite concentration amongst clinical phenotypes may be linked to biological events that provide clues to the pathophysiology of disease. Metabolomics is an ideal technology to apply in CF, as it allows for a qualitative and quantitative characterization of the pathobiology driven by CFTR mutations. It has been well-established that patients with CF who hold the same CFTR mutations are often clinically heterogeneous, making individualized approaches to clinical care vital to the preservation of lung function. Therefore, it is likely that a profile, or panel, of biomarkers is more likely to reflect disease severity rather than one single parameter.¹¹ Metabolomic profiling provides the technology by which to identify biomarkers of metabolism in a biological system, serving as the ideal platform to investigate the mechanisms underlying CF pulmonary exacerbation.

Previous cross-sectional metabolomics work in respiratory disease has focused on providing diagnostic clarity or generating biochemical clues to disease progression. Nuclear magnetic resonance (NMR) based metabolomics of exhaled breath condensate (EBC) discriminates asthmatic children and adults with chronic obstructive pulmonary disease from healthy controls, in addition to subjects with CF from those with primary ciliary dyskinesia.^{12–15} In CF, metabolomics analysis has identified metabolites in biological fluids for hypothesis

generation and additional cross-sectional analysis of EBC using NMR discriminates stable and unstable CF.^{16–20} However, no longitudinal work has been done applying this technology to CF pulmonary exacerbation utilizing subjects as their own control. The objective of this study was to use global, untargeted metabolomic profiling to identify new metabolite biomarkers of CF pulmonary exacerbation in plasma from CF patients. Some of the results of this study have been previously reported in the form of an abstract.²¹

Materials and Methods

Study Design

The subjects used in this analysis were taken from a larger cohort study of CF pulmonary exacerbations. In brief, a single-center, prospective 2-year longitudinal cohort study of patients with CF during times of pulmonary exacerbation (hospitalization) and times of clinical stability (outpatient clinic visits) was performed. For study purposes, a pulmonary exacerbation was defined as the need for hospitalization for intravenous (IV) antibiotics and airway clearance for an increase in pulmonary symptoms and/or a 10% decrease in FEV₁. Clinical stability was defined as no current use of IV antibiotics or lack of symptoms consistent with a pulmonary exacerbation. All hospitalized patients received standard of care therapies. Each subject provided a blood sample and underwent pulmonary function testing approximately within 24 hrs of initiation of IV antibiotics (initial sample), on day 3–4 (interim sample) and on day 5–7 (final sample). Upon discharge, all subjects were followed at quarterly outpatient clinic visits for 2 years where plasma samples and pulmonary function testing data were collected. Subjects were not uniformly fasting at the time of sample collection. The study was approved by the University of Minnesota Institutional Review Board (IRB#0809M45601) and informed consent and/or assent were obtained from each of the subjects and/or their parents or guardians.

For purposes of this analysis, 25 patients with a confirmed diagnosis of CF identified on hospitalization for a pulmonary exacerbation and enrolled in this larger cohort study of CF pulmonary exacerbation were selected for this study.²² A matched pair of plasma samples, one during pulmonary exacerbation and one during an outpatient clinic visit, was selected for discovery metabolomic profiling (n = 50 plasma samples). Those samples selected for global metabolomic profiling were those with the largest measured change in FEV₁ percent predicted between hospitalization and a subsequent clinic visit.^{23,24}

Patient Plasma Samples

Blood was obtained in standard BD Vacutainer EDTA tubes. Serum and plasma were separated by centrifuge using a standard operating laboratory procedure. Plasma was immediately frozen and stored at –80°C until it was shipped on dry ice to Metabolon, Inc. (Durham, NC) for metabolomic analysis.

Metabolomic Profiling Platform

In collaboration with Metabolon, Inc. (Durham, NC), the biochemical profiles of plasma samples were analyzed using both gas chromatography (GC) and liquid chromatography (LC) platforms coupled with mass spectrometry (MS). Sample preparation was conducted

using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The LC/MS/MS experiments were performed in both positive and negative ion modes to maximize the identification of metabolites with diverse chemical properties. Quality control was assessed by the use of blanks, derivatization standard, internal standard, and recovery standard within each run. Compounds were identified by comparison to Metabolon, Inc.'s available library of purified standards.

Liquid Chromatography/Mass Spectrometry (LC/MS, LC/ MS2)—The analysis was performed using a Waters ACQUITY UPLC (Waters Corporation, Milford, MA) and a Thermo-Finnigan LTQ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) using both an acidic positive ion optimized conditions (gradient eluted using water/methanol containing 0.1% formic acid) and a basic negative ion (gradient eluted using water and methanol containing 6.5 mM ammonium bicarbonate) conditions. For ions with counts greater than 2 million, an accurate mass measurement could be performed. The typical mass error was <5 ppm.

Gas Chromatography/Mass Spectrometry (GC/MS)—The analysis was performed using a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Analyzed samples underwent vacuum desiccation (minimum 24hrs) followed by derivatization under nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA).

Metabolite Identification—Metabolites were identified by the automated comparison of the ion features discovered in the patient plasma samples to a reference library of chemical standards. These standards were run under the identical conditions and their retention time, molecular weight, adducts and in-source fragments as well as their associated MS/MS spectra were recorded to allow for rapid identification of metabolites in patient samples with high-confidence. At the time of the analysis, more than 1,000 commercially available purified standard compounds were available in Metabolon Inc.'s reference library.

Statistical Analysis

To test for associations between metabolite levels and exacerbation status, mixed effects models were fit to the logarithm of metabolite levels. These models had indicator variables for exacerbation status and controlled for body mass index (BMI) and FEV₁ percent predicted (so the regression coefficient for the indicator variable is approximately equal to the log of the fold change in metabolite expression due to an exacerbation). Controlling for FEV₁ percent predicted is essential as this variable was used to select the subsample that was analyzed. The mixed effects models had subject specific random effects to model the similarity of measurements coming from the same subject. Parameter estimates were obtained using restricted maximum likelihood and Wald tests were used to test null hypotheses. Given the large number of metabolites investigated *P*-values were adjusted using the Benjamini Hochberg method and these values were compared to 0.05. To visualize the high dimensional data, principal components analysis was used on a subset of the data utilizing those metabolites that differ between the groups with an FDR (false discovery rate)

of 5%. All calculations were conducted using R version 3.1.0 and the nlme package was used to fit the mixed effects models.

Results

Study Population

Table 1 describes the demographics of the 25 CF subjects. The mean change in FEV₁% predicted between pulmonary exacerbation and outpatient clinic was 15.6% (95%CI: 13.8–17.4).

Metabolomic Profile

398 metabolites were identified in CF plasma. Upon adjustment for multiple comparisons via control of the FDR while controlling for confounders, five metabolites were found to differ between CF patients in pulmonary exacerbation compared to a clinically stable state ($P < 0.05$, $q < 0.05$ for all metabolites listed except for hypoxanthine, Table 2).

Metabolites in CF Pulmonary Exacerbation

The differentially expressed metabolites can be grouped into four categories based on the biochemical pathways involved.

Nucleotide and Nucleoside Metabolism—Hypoxanthine, a metabolite involved in purine biosynthesis and nucleotide metabolism, was decreased during a CF pulmonary exacerbation (fold change 0.605, $P = 0.0100$, $q = 0.2211$, Fig. 1). Plasma xanthine concentration was not significantly different, although it demonstrated a similar decrease. N4-acetylcytidine, a modified nucleoside metabolite of pyrimidine metabolism and the product of the degradation of transfer ribonucleic acid (tRNA), was also significantly decreased during a CF pulmonary exacerbation (fold change 0.681, $P = 3.00 \times 10^{-0.4}$, $q = 0.0291$, Fig. 1).

Amino Acid Metabolism—N-acetylmethionine (NAM) is involved in methionine and taurine metabolism and was significantly decreased in the plasma of CF patients during exacerbation (fold change 0.704, $P = 1.70 \times 10^{-0.4}$, $q = 0.0219$, Fig. 2).

Carbohydrate Metabolism—Sugars play an important function in cellular metabolism and serve as important precursors of nucleotides and fatty acids. Metabolomic profiling revealed plasma mannose concentration significantly decreased during a pulmonary exacerbation (fold change 0.531, $P = 1.25 \times 10^{-0.4}$, $q = 0.0219$, Fig. 3).

Steroid Metabolism—Cortisol is a steroid hormone that is released by the adrenal cortex in response to stress. The plasma concentration of cortisol was significantly decreased during a CF pulmonary exacerbation (fold change 0.528, $P = 6.34 \times 10^{-0.4}$, $q = 0.04922$, Fig. 4).

Principal Components Analysis

Principal components analysis was performed to determine the best explanation of the variance in the dataset. The greatest variance lies in the first principal component, with the next greatest variance found in the second principal component. PCA utilizing the five metabolites identified as significantly different between a pulmonary exacerbation and clinically well state (hypoxanthine, N-acetylmethionine, N4-acetylcytidine, mannose and cortisol) provided good separation between the two phenotypes as 79% of the variation of the data can be explained in the first two principal components (Fig. 5).

Discussion

The link between pulmonary exacerbation, lung function decline and lung disease progression in CF remains poorly defined. To gain insight into the biochemical mechanisms of CF pulmonary exacerbation, we used an untargeted metabolomics platform to compare the plasma metabolite profiles of patients during an exacerbation with a clinically stable state. Each patient served as their own control, providing the ideal cohort in which to utilize discovery metabolomics technology. We identified five metabolites that provide insight into the molecular mechanisms of CF pulmonary exacerbation and also provide putative biomarkers which can be validated in future cohort studies.

Nucleotide and Nucleoside Metabolism

We reported hypoxanthine given its potential biologic importance in CF lung disease even though it was not identified using an FDR <5% (FDR <25%). Hypoxanthine concentration was increased in BALF from lobes of the lung containing localized bronchiectasis in preschool children with CF and found to correlate with neutrophil count and important clinical outcomes in CF.^{16,25} Similar to our finding it was decreased in the primary human bronchial epithelial cells of CF subjects.¹⁷ Extracellular purine concentration in airway samples is thought to be a biomarker of inflammation in CF^{26,27} while hypoxanthine has been reported as a marker of oxidative stress.^{17,28,29} Hypoxanthine is formed secondary to ATP degradation, and its conversion to uric acid is facilitated by the enzyme xanthine oxidase, generating free oxygen radicals.³⁰ Associated metabolites (i.e. xanthine, inosine, ADP, and AMP, data not shown) trended toward a decreased plasma concentration in our study although this did not reach statistical significance. A decreased hypoxanthine concentration may be secondary to its increased conversion to uric acid during exacerbation, generating superoxide and hydroxyl radicals and resulting in cellular damage, although further investigation is needed.

We found N4-acetylcytidine concentration decreased during a pulmonary exacerbation, similar to a finding in the primary human bronchial epithelial cells of CF subjects.¹⁷ N4-acetylcytidine is a modified nucleoside that is produced in the degradation of transfer ribonucleic acid (tRNA) and excreted into the urine. Although it has been described as a urinary marker for colorectal cancer,³¹ its significance in the pathophysiology of CF pulmonary exacerbation requires further investigation.

Amino Acid Metabolism

The plasma concentration of a methionine derivative, N-acetylmethionine (NAM), was decreased during CF pulmonary exacerbation. Associated metabolites (i.e. cysteine, S-methylcysteine, N-formylmethionine, data not shown) also demonstrated a similar trend. Methionine, an essential amino acid, is critical in maintaining methylation and the redox state of cells.³² NAM is also involved in taurine metabolism. Taurine is the major intracellular amino acid and participates in osmoregulation, membrane protection, antioxidant defense and the regulation of cellular calcium homeostasis.³³ Taurine was detected in the BALF of CF subjects with high amounts of airway inflammation¹⁶ and NAM is considered to be an antioxidant agent itself.³⁴ Given its role as a precursor to glutathione, its decreased concentration may signify the potential for oxidative stress.

Carbohydrate Metabolism

Plasma mannose concentration was decreased during CF pulmonary exacerbation. We observed similar trends in associated metabolites involved in carbohydrate metabolism (i.e. fructose, mannitol, sorbitol, and methyl-beta-glucopyranoside, data not shown). Mannose is a C-2 epimer of glucose, a sugar monomer important in the glycosylation of numerous proteins in addition to serving as a substrate for the glycolytic pathway. Glucose metabolism was previously found to be suppressed in CF human bronchial epithelial cells, with mannose concentration noted to be significantly decreased.¹⁷ Mannose is an essential component of the glycoconjugates present in a wide variety of bacterial, viral, and fungal pathogens that serve as the binding site for mannose-binding lectin (MBL), an important part of the innate immune system.³⁵ Mannose concentration may be decreased in illness secondary to its utilization to defend the lung from the increased bacterial burden seen during a CF pulmonary exacerbation.³⁶ Decreased concentrations may also be secondary to increased uptake into the glycolytic pathway during the catabolic state of a CF pulmonary exacerbation.

Steroid Metabolism

The finding of a decreased plasma cortisol concentration during a CF pulmonary exacerbation was surprising. Cortisol is released in response to stress and functions to increase blood sugar, suppress the immune system, curb inflammation, and aid in nutritional metabolism. Plasma cortisone concentration was also decreased, although this finding was not significant. A number of androgens involved in steroid metabolism (i.e. 7-beta-hydroxycho-lesterol, epiandrosterone sulfate, androsterone sulfate, and lanosterol, data not shown) demonstrated a similar trend. A decreased plasma cortisol suggests the possibility of adrenal suppression and impaired adrenal responses have been shown to be common in patients with non-CF bronchiectasis.³⁷ The mechanism for this in CF is intriguing and should be investigated further.

Our longitudinal study is the first to examine the change in metabolomic profiles in patients with CF that occur during a pulmonary exacerbation, utilizing each subject as their own control. Taken individually, the metabolites provide a novel look at the derangements in metabolism that occur during a CF pulmonary exacerbation. As a panel, principal component analysis provided good separation between the two clinical states. Our study is

not without limitation. Metabolite identification is limited by Metabolon's library of available standards. The cohort of patients selected for analysis may also limit the generalizability of our findings, given the subjects were older with more established lung disease. Finally, the blood samples were not collected during a fasting state which may have an effect on the concentration of the measured metabolites (i.e. mannose). However, the finding of five metabolites with significant differences and the ability to discriminate between an exacerbation and well state provides additional insights into CF lung disease. To provide a more comprehensive understanding of CF pulmonary exacerbation, future studies should incorporate other established markers of CF lung disease into the analysis. Such work should include the measurement of inflammatory markers, isoprostanes and markers of oxidative stress found in EBC, utilizing established non-invasive techniques.^{38–41} The investigation of relationships between metabolite biomarkers with systemic markers of inflammation would also broaden the scope and provide valuable insight into mechanisms of CF pulmonary exacerbation.^{42,43} This data can serve as the foundation for future hypothesis-driven clinical research and provide potential outcome measures for novel therapies and treatments.

Acknowledgments

T.A.L. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, including and especially any adverse effects. In addition, T.A.L. conceived of and designed the study, contributed to the analysis and interpretation of the data and wrote the manuscript; C.S. R. contributed to the data analysis and statistical interpretation and participated in writing the manuscript; C.B.W. performed sample analysis and data acquisition; C.B. performed sample analysis, assay development and data acquisition; C.H.W. contributed to the study design, oversaw data acquisition and critically reviewed the manuscript. All the authors contributed to the writing of the manuscript. The funding sources for this study did not have a role in study conception, design, conduct and/or analysis and did not modify or approve this manuscript. The authors wish to thank Metabolon, Inc. for their collaborative metabolomics work and Kyle Brandy, MS for his contribution to the collection and processing of plasma samples from study subjects. We also sincerely thank the CF patients and families for their willingness to participate in our research study.

Funding source: Cystic Fibrosis Foundation; Number: CFF LAGUNA08A0, National Institutes of Health; Numbers: K12HD068322, 1U01HL081335-01, M01RR00069.

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Abbreviations

ARDS	Acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
BMI	Body mass index
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
EBC	Exhaled breath condensate
FDR	False discovery rate
FEV1	Forced expiratory volume in one second
GC	Gas chromatography
IV	Intravenous antibiotics
LC	Liquid chromatography
MBL	Mannose binding lectin
MS	Mass spectrometry
NAM	N-acetylmethionine
NMR	Nuclear magnetic resonance
PCA	Principal components analysis
tRNA	Transfer ribonucleic acid

Fig. 1.

Nucleotide and Nucleoside Metabolism: (A) Plasma concentration of hypoxanthine is decreased during CF pulmonary exacerbation (SICK) compared to outpatient clinic (WELL) (fold change 0.605, $P = 0.0100$, $q = 0.2211$). (B) Plasma concentration of N4-acetylcytidine is also decreased during CF pulmonary exacerbation (fold change 0.681, $P = 3.00 \times 10^{-0.4}$, $q = 0.0291$). ○ = data point outside of the 1.5 intra-quartile range.

Fig. 2.

Amino Acid Metabolism: Plasma concentration of N-acetylmethionine is decreased during CF pulmonary exacerbation (SICK) compared to outpatient clinic (WELL), (fold change 0.704, $P = 1.70 \times 10^{-0.4}$, $q = 0.0219$). ○ = data point outside of the 1.5 intra-quartile range.

Fig. 3. Carbohydrate Metabolism: Plasma concentration of mannose is decreased during CF pulmonary exacerbation (SICK) compared to outpatient clinic (WELL), (fold change = 0.531, $P = 1.25 \times 10^{-0.4}$, $q = 0.0219$). ○ = data point outside of the 1.5 intra-quartile range.

Fig. 4. Steroid Metabolism: Plasma concentration of cortisol is decreased during CF pulmonary exacerbation (SICK) compared to outpatient clinic (WELL), (fold change = 0.528, $P = 6.34 \times 10^{-0.4}$, $q = 0.0492$). ○ = data point outside of the 1.5 intra-quartile range.

Fig. 5. Principal components analysis utilizing our five identified metabolites shows good separation between a pulmonary exacerbation state (SICK) and a clinically stable state (WELL). ○ = WELL, △ = SICK.

Table 1
Demographics of Sub-Cohort of Subjects Selected for Plasma Metabolomics Analysis

Characteristics	Values (range)
Subjects	25
Hospital Admissions	25
Clinic Visits	25
Male Subjects	11
Age (years)	27.0 (13.6–38.5)
Clinic FEV ₁ (L)	2.6 (1.3–4.1)
Clinic FEV ₁ (% predicted)	69 (38–105)
Exacerbation FEV ₁ (L)	2.1 (0.8–3.6)
Exacerbation FEV ₁ (% predicted)	55 (23–82)
Overall FEV ₁ (L)	2.3 (0.8–4.1)
Overall FEV ₁ (% predicted)	64.5 (23–105)
BMI (kg/m ²)	21.6
F508del/F508del	14
F508del/other	11

Values are presented as number or median (range).

Table 2
Panel of Metabolites Significantly Different in CF Pulmonary Exacerbation

Metabolite	Biochemical Pathway	Fold Change	During CF Pulmonary Exacerbation	Method of Detection
Hypoxanthine	Nucleotide, Purine Metabolism	0.605	Decreased	LC/MS neg
N4-acetylcytidine	Nucleoside Metabolism	0.681	Decreased	LC/MS neg
N-acetylmethionine	Amino Acid, Methionine Metabolism	0.704	Decreased	LC/MS neg
Mannose	Carbohydrate Metabolism	0.532	Decreased	GC/MS
Cortisol	Steroid Metabolism	0.528	Decreased	LC/MS pos

All Metabolites With $P < 0.05$ and $q < 0.05$, Except for Hypoxanthine. LC: Liquid Chromatography; MS: Mass Spectrometry; GC: Gas Chromatography; neg = negative Ion mode; pos = positive ion mode.