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Metabonomics reveals altered metabolites related to inflammation and energy utilization at recovery of cystic fibrosis lung exacerbation

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ABSTRACT

Background: Cystic fibrosis lung disease is characterized by chronic bacterial infections in the setting of mucus abnormalities. Patients experience periodic exacerbations that manifest with increased respiratory symptoms that require intensification of therapy with enhanced airway clearance and intravenous (IV) antibiotics.
Objectives: In an observational study we tested if the profile of metabolites in serum distinguished the pre-from post-exacerbation state and which systemically measurable pathways were affected during the process to recovery.
Methods: Serum collected within 48 h of start and completion, respectively of IV antibiotics was collected from people with CF ages 6–30 years. Three day food records were collected prior to each sample. To reduce variation between subjects only subjects who had pancreatic insufficiency, had similar CF mutations, and did not have CF liver disease or diabetes were included. Metabolomic profiling was conducted by Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy with metabolites being identified based on retention time/index, mass to charge ratio and comparison to known compounds. Biostatistical analyses used paired *t*-test with correction for multiple comparisons and orthogonal partial least square discriminant analysis.
Results: Thirty subjects (20 male) with a mean ± SEM age of 15.3 ± 1.2 years participated, 17 of whom had matched food-records. Lung function was significantly improved post-therapy compared to pre-therapy, (mean ± SEM) 75 ± 4% vs. 68 ± 4% predicted (n = 26). Serum metabonomics showed distinction of the pre-vs. post-therapy groups with 123 compounds contributing to the differentiation pre-versus post-antibiotics by multiple biostatistical analyses. Compounds and pathways affected included bile acids and

Abbreviations: CF, cystic fibrosis; CFTR, Cystic Fibrosis Transmembrane Regulator; CRP, C-reactive protein; IV, intravenous; UNC, University of North Carolina at Chapel Hill; FEV_1, forced expiratory volume in 1st second; RI, retention time/index; UPLC, ultrahigh performance liquid chromatography-tandem mass spectroscopy; ESI, electrospray ionization; QC, quality control; FDR, false discovery rate; q, significance at a 5% FDR cut-off; OPLS-DA, orthogonal partial least square discriminant analysis; VIP, variable influence on projection score; BA, bile acids; BHBA, 3-hydroxybutyrate; AA, arachidonate; DHA, docosahexaenoate; n3-DPA, docosapentaenoate; *ARG*, arginase; *ODC*, ornithine decarboxylase; *NOS*, nitric oxide synthase; IDO, indoleamine-2-3-dioxygenase.

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microbial derived amino acid metabolites, increases in lipid classes of the glycerophospholipid, glycerolipids, cholesterol, phopsholipids, and most pronounced, the class of sphingolipids. Changes in n6/n3 fatty acids, decreased polyamines but increased metabolites in the nitric oxide pathway, and changes in the tryptophan-kynurenine pathway indicated decreased inflammation at resolution of exacerbation.
Conclusions: Changes in serum metabolites that distinguished CF pulmonary exacerbation vs. resolution of symptoms showed evidence of decreased inflammation and improvement from a catabolic state.

1. Introduction

Cystic fibrosis is a systemic disease caused by abnormal processing and function of the ion channel Cystic Fibrosis Transmembrane Regulator (CFTR). The most frequent genetic mutation is F508del resulting in absence of functional CFTR. Defects in regulating chloride and bicarbonate secretion in secretory cells lead to abnormally viscous secretions [1]. The classic disease manifestations are chronic bacterial lung infections, pancreatic insufficiency and development of CF diabetes, liver disease and other complications with increasing age. Despite knowledge of the genetic and protein defect the etiology of several other manifestations, for example the excessive, neutrophil dominated airways inflammation and abnormal levels of essential fatty acids remains debated [2].

A characteristic feature of CF lung disease are pulmonary exacerbations presenting with increased cough, sputum expectoration, decrease in lung function and weight loss. Pulmonary exacerbations are associated with progression of disease, costs to the healthcare system, and disruption of patients' lives with lower quality of life. The triggers and pathophysiology of exacerbations and their resolution are still incompletely understood. Appearance of new infecting organisms, change in microbiome, or virus infections do not fully explain these events [3–6]. In older patients with more advanced disease changes in inflammatory biomarkers are detectable in blood, e.g. C-reactive protein (CRP) [7,8], and blood mRNA biomarkers [9], indicating a systemic response; however these conventional inflammatory markers are rarely detectable in younger patients with milder disease [10,11]. Yet, markers of exacerbations that were detectable in serum would provide additional diagnostics in this population.

Metabonomics allows simultaneous and systematic profiling of metabolite levels in organisms in response to environmental influences, ageing, or disease [12]. Targeted and untargeted metabolomics/metabonomics in CF has been applied to CF respiratory samples and blood in various scenarios [13,14]. Analyses of respiratory secretions showed elevated markers of inflammation including purines [15] and lipid mediators [16]. Although not directly measuring changes in the lung, an advantage of blood based biomarkers is their easier collection and availability at all ages compared to respiratory secretions. Further, systemic markers may provide insight into pathophysiology of exacerbations. We had shown that serum metabolite profiling differentiated samples CF vs. non-CF in children, i.e. at an early stage of disease [14]. Recently serum metabolomics profiling was used to ascertain changes after 6 months of CFTR corrector therapy, which revealed bile acids, phosopholipids and amino acid alterations as main discriminatory compounds [17]. A study during the early stages of metabonomics evaluated metabolites in plasma at time of CF exacerbation compared to samples obtained at a subsequent clinic visits. Four compounds were differentially expressed, i.e. N-acetylmethionine, mannose, N4-acetylcytidine, and cortisol with trends for changes in hypoxanthine [18]. Another study evaluated the effect of high dose vitamin D therapy in addition to intravenous (IV) antibiotics during pulmonary exacerbations on metabolites in plasma of adults with CF [19]. Pathway enrichment analyses indicated differences in omega-3 fatty acids, glycosphingolipids, phosphatidylinositol phosphate, pyrimidine, urea cycle/amino group and glycolysis pathways between vitamin D and placebo groups but authors did not comment on changes at exacerbation state compared to samples collected one week into treatment.

The current study aimed to compare short term changes between serum collected at start versus completion of IV antibiotics to test the hypothesis that metabonomic profiles differentiate the acute exacerbation state from that at resolution of exacerbation. Further we evaluate which metabolites and network of metabolites are altered that could be tested as bio-markers in future studies.

2. Methods

2.1. Subjects and sample collection

People with CF ages 6–45 years with at least one mutation of F508del and pancreatic insufficiency attending the CF center at the University of North Carolina at Chapel Hill (UNC) were eligible. Exclusion criteria were diabetes, CF liver disease (abnormal synthetic function or elevation of transaminases), or having undergone organ transplant. These inclusion criteria were selected to make our cohort representative of people with CF in the U.S., i.e. ~86% have at least one F508del mutation [20,21]. Exclusion of subjects with CF diabetes and CF liver disease reduced confounding by these complications. Subjects were enrolled at time of admission to the hospital for a respiratory exacerbation between August 2012 and February 2015. Exacerbations were defined as increasing respiratory symptoms for >3 days and decrease in lung function. Pre-antibiotic samples were collected within 24 h of admission and post-antibiotic samples within 48 h of the last antibiotic dose. Treatment duration was determined by the treating physician unrelated to this study. Typically IV therapy is given for 2–3 weeks. Blood was collected after an overnight fast to reduce influence of post-prandial and absorption related metabolic changes, into serum separator tubes without additives. The blood was allowed to clot at room temperature for 30 min and then spun at 1500×*g* for 15 min. Aliquots were stored at −80 °C until processing for metabolomics.

2.2. Food diaries

Food diaries were collected for the 3 days prior to each blood sample; i.e. for the 3 days prior to admission and the last 3 days of IV antibiotics. Dietary contribution of fat, carbohydrates, protein, vitamins and trace elements were analyzed using nutrition analysis software (Food Processor® 10.15.0).

2.3. Clinical data

All clinical information was obtained at time of sample collection. Lung function expressed as forced expiratory volume in 1st

second (FEV_1) was obtained per ATS-ERS criteria and expressed as % predicted for race, gender, and height [22,23].

2.4. Metabonomic data processing

Samples were prepared and analyzed by Metabolon® as described previously [24,25] using an untargeted metabolomic technology platform based on Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy. Briefly, proteins were precipitated from 100 μl of human serum with methanol containing 4 standards to monitor extraction efficiency. The resulting extract was divided into four aliquots that were optimized for hydrophilic vs. hydrophobic and positive and negative ion mode electrospray ionization, respectively. Technical controls included a pooled matrix sample generated by taking a small volume of each experimental sample which served as a technical replicate throughout the data set; extracted water samples as process blanks; and a cocktail of quality control (QC) standards as positive controls.

2.5. Metabolite identification and quantification

Raw data was extracted, peak-identified and QC processed using Metabolon's® hardware and software. Metabolites were matched to authenticated standards in Metabolon's® library that contains the retention time/index (RI), mass to charge ratio, and chromatographic data (including MS/MS spectral data). Biochemical identifications were based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library ± 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. Peaks were quantified using area-under-the-curve.

2.6. Statistical analyses

Descriptive statistics on clinical and nutritional data were performed in JMP-Pro 12.0.1. (SAS Institute Inc., Cary, NC).

Data were filtered to include metabolites that were present in ≥80% of samples at each time point. Principal component analysis (PCA) as unsupervised classification method and orthogonal partial least square discriminant analysis (OPLS-DA) as supervised classification method were used for initial analyses. OPLS-DA seeks to identify a panel of metabolites that can best separate the classes based on differences pre-vs. post therapy [26]. The default 7-round cross-validation in SIMCA (Umetrics, Umeå, Sweden) package was applied with 1/7 of the samples being left out from the mathematical model in each round. Besides cross validation, permutation based validation was also used to prevent overfitting in SIMCA. 999 permutated data sets were generated. OPLS-DA was performed and Q2Y value was calculated for each permutated data set. The separation was considered significant if the Q2Y values from the original data set was found to be higher than 95% of the Q2Y values (i.e. $P < 0.05$) generated from the permutated data sets (Suppl. Figure 1). Variable Influence on Projection (VIP) score was calculated based on the PLS weights and the variability explained in OPLS-DA. Metabolites with VIP>1 were considered the important metabolites responsible for the differentiation between samples from the pre and post antibiotics treatment time points. Shapiro-Wilk test on log transformed data was used for normality check. Paired-t-test was performed on log (base 2) transformed data to identify metabolites that were significantly different between the two time points. Metabolites with non-normal distribution were also analyses by Wilcoxon signed rank test. Correction for multiple comparisons was applied at a cutoff of 5% false discovery rate (FDR at $q < 0.05$) [27]. Ratios between metabolites were calculated at the patient level, log (base2) transformed and analyzed by paired *t*-test but were not FDR corrected as we focused on single networks.

Power calculations were based on use of paired *t*-test and using variance from our prior cohort study [14]. Estimates were derived under the assumptions of an effect size is 1.2 (or 1), and 10000 simulations on normally distributed data, which demonstrated the potential to discern 46 true differences at 5% FDR using a paired *t*-test on 30 patients.

2.7. Ethics statement

The study was approved by the IRB/Ethics Review Board of the University of North Carolina and all subjects or parents provided written consent and minors provided additional assent.

3. Results and Discussion

3.1. Subject characteristics

Thirty subjects (20 male) had pre- and post-antibiotic serum samples collected. Twenty-three subjects were F508del homozygous and the remainder had one F508 del and another mutation resulting in lack of functional CFTR. Mean ± SEM age was 15.3 ± 1.2 years ranging from 6 to 30 years and ten subjects were >18 years. The mean duration of antibiotics was 16 ± 1 days. As frequently seen in CF, many subjects had multi-organism infection. The most frequent bacteria cultured, single or in combination, from the respiratory secretions were *P. aeruginosa* (n = 20) oxacillin sensitive *Staphylococcus aureus* (n = 11) and oxacillin resistant *Staphylococcus aureus* (n = 11). Beta-lactams (n = 23) and IV aminoglycosides (n = 12) were the most frequently used antibiotic classes and 23/30 subjects were on more than one antibiotic.

Not all subjects completed lung function, expressed as forced expiratory volume in 1 s (FEV_1) at both time points for various reasons. In the 26 subjects with paired measurements, FEV_1 was significantly improved after therapy (Table 1) yet three subjects did not achieve at least 90% of their baseline FEV_1 at end of therapy. Two subjects were deemed not be clinically recovered to baseline despite recovery of lung function and three subjects did not complete spirometry post-therapy but were clinically deemed as recovered to baseline. The increase in BMI was not significant, possibly explained by the fact that 13/29 subjects showed loss of weight or less than a 0.5 kg increase.

3.2. Metabonomic signature differentiates pre- and post-antibiotic therapy states

Of the 690 identified metabolites i.e. those with validated standards in the library, 122 compounds (including 13 drugs/drug metabolites) were missing more than 20% at both time points and were removed from analyses. Among the remaining 568, 198 compounds were differentially expressed in the pre-compared to post antibiotic samples at a raw p-value of 0.05 (Supplemental File 1). After adjusting for multiple comparisons at a false discovery rate (FDR) of 5% 170metabolites remained significantly different. The majority were increased at end of therapy.

Principal component analyses showed no distinction by age, number of F508 del mutations, gender, FEV_1 or response to therapy. Orthogonal Partial Least Squares Discriminant Analysis showed clear discrimination of pre-vs. post-antibiotic samples (Fig. 1). In this analysis 210 metabolites were significant i.e. a Variable Influence on Projection score (VIP) > 1(S Fig. 1).

Comparison of the 210 metabolites derived from OPLS-DA and the 170 metabolites derived from the paired *t*-test showed high consistency of the analyses. Additional analyses of the 28 non-

Table 1
Key clinical parameters.

Parameter Mean ± SEM	All pre-antibiotic	All post-antibiotic	Paired pre-antibiotic	Paired post-antibiotic	p-value (paired n subjects)
FEV_1% predicted	68 ± 4 (n = 27)	75 ± 4 (n = 27)	68 ± 4	76 ± 4	0.0005 (n = 26)
BMI (kg/m^2)	18.7 ± 0.8 (n = 30)	19.0 ± 0.8 (n = 30)	18.7 ± 0.8	19.0 ± 0.8	0.115 (n = 30)
Calorie intake (kcal/day)	2265 ± 229 (n = 21)	2621 ± 250 (n = 22)	2273 ± 213	2701 ± 296	0.08 (n = 17)
Protein (g) (% of calories)	83 ± 9 (15 ± 1)	101 ± 11 (16 ± 1)	89 ± 10 (15 ± 1)	107 ± 13 (15 ± 1)	0.22
Fat (g) (% of calories)	94 ± 11 (36 ± 1)	108 ± 11 (37 ± 1)	95 ± 11 (37 ± 1)	113 ± 14 (37 ± 1)	0.13
Carbohydrates (g) (% of cal.)	276 ± 28 (49 ± 2)	318 ± 31 (49 ± 2)	270 ± 22 (49 ± 2)	320 ± 36 (49 ± 2)	0.26

Footnotes: FEV_1% = Forced expiratory volume in 1st second expressed as % predicted for race, gender, and height [23]. BMI = body mass index. Data are rounded to clinical relevance.

normally distributed metabolites by non-parametric Wilcoxon signed rank test resulted in 123 compounds contributing to the differentiation pre-versus post-antibiotics by all methods. Metabolites included those linked to bacterial metabolism, bile acids, lipid metabolism (cholesterol, sphingolipids and phospholipids), and inflammation/anti-oxidants (arachidonic acid pathway, polyamines, and retinol).

3.3. Effects of antibiotics on gut microbiome and bile acids

After antibiotic therapy several metabolites of phenylalanine and tyrosine derived from microbial metabolism of non-digestible peptides were decreased. (Table 2).

These findings are consistent with alterations of the gut microbiome by antibiotics, which are reflected in the serum metabonomic signature. Dimethyl sulfone was also lower (0.089 fold, q = 0.04) at end of antibiotics. This compound can originate from microbial metabolism of methione in the gut [33] and changes could indicate increased fat intake during IV therapy and/or changes in bacterial flora. Supporting such changes in serum metabolites are findings from animal models treated with broad-spectrum antibiotics that showed altered intestinal microbiota accompanied by significant decreases in metabolites of phenylalanine and tyrosine in serum [34]. Experimental studies in healthy human volunteers given oral vancomycin also showed significant shifts in fecal microbiota with concomitant increases in fasting, plasma total bile acids [35,36].

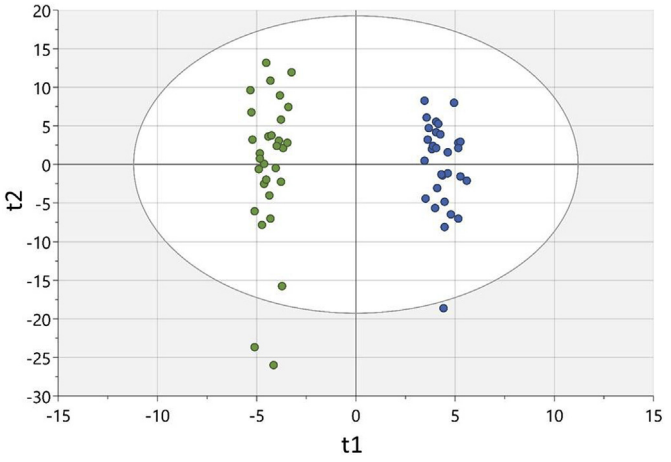

Fig. 1. OPLS-DA separates samples between pre (green) and post (blue) antibiotics treatment. T1 and t2 are the first two latent variables identified by OPLS-DA to distinguish the two time points. Each of them is a linear combination of all of the metabolites. R2Y = 0.982 and Q2Y = 0.308 indicate that the model has good power to discriminate samples taken at the two time points. This model passed permutation based validation to rule out overfitting (Fig. S1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Gut microbes are essential to production of secondary bile acids in the gut [36]. After antibiotic therapy several secondary bile acids were decreased, e.g. glycolithocholate sulfate and the conjugated primary bile acid taurochenodeoxycholate was increased. The bile acid precursors 3β,7α-dihydroxy-5-cholestenoate and 7-α-OH-3-oxo-cholestenoate (7-HOCA) were increased pointing towards higher bile acid synthesis at recovery.

The sulfated bile acids, glycolithocholate sulfate (0.74×, q = 0.03) and glycocholenate sulfate (0.88×, q = 0.02) were lower post therapy. Sulfation of bile acids is a mechanism unique to human bile acid circulation that is enhanced during cholestasis [37]. Cystic fibrosis increases the risk of cholestasis thus decreases in sulfated bile acids may indicate improved bile acid flow post therapy. Such findings are consistent with results of a therapeutic trial of ursodeoxycholic acid in obese subjects that showed not only the expected improvement in transaminitis but also lower serum levels of hippurate, and decreases in phenylalanine metabolites similar to those seen in our study [29].

Jointly these findings suggest that antibiotic therapy and clinical improvement is associated with loss of secondary bile acids due to decreased conjugation and enterohepatic circulation with increased bile acid synthesis and increased bile flow.

3.4. Increased lipid pool and changes in inflammatory lipid mediators

Increases post-therapy were noted for several groups of lipids (i.e. glycerolipids, sterol, phopsholipids, glycerophospho-lipids, and the complex lipids of the sphingolipids class).

Increases in the energy providing monoacylglycerols and diacylglycerols (e.g. 1-myristoylglycerol (5.66×, q = 0.005), 1-linolenoylglycerol (18:3) (6.0×, q = 0.04) or 1-palmitoyl-2-linoleoyl-glycerol post-therapy could indicate increased availability (dietary intake or absorption) or/and changing triglyceride use, i.e. lower metabolic needs with resolution of symptoms. (Table 3). In a prior study we had seen evidence of lower β-oxidation of fatty acids in CF compared to non-CF children [14], with decreases in the ketone body 3-hydroxybutyrate (BHBA) and medium and short chain carnitines in CF. Here we saw increases in medium and branched chain acyl-carnitines as a class post therapy but 3-hydroxybutyrate (BHBA) and carnitine were not significantly different post therapy. Jointly the elevations in acyl-carnitines, which are involved in β-oxidation of fatty acids, and BHBA are suggestive of increased oxidation of fatty acids. These changes suggest that improvements in infection/inflammation are associated with enhanced β-oxidation and increased energy production [38].

Weight gain typically accompanies resolution of pulmonary exacerbations in CF. It has been shown that this is related to decreased energy expenditure as weight gain correlated with changes in resting energy expenditure but not with calorie intake [39]. Our data of increasing lipid availability with non-significant

Table 2
Amino acid metabolites potentially derived through microbial activity.

Name of metabolite	Fold change and q value	Function/relevance	Source aminoacid/Pathway
Hippurate	0.89× q = 0.0060	Alterations similar to inflammatory bowel disorders [28]. Ursocholate therapy associated with increased bile flow and decreases in hippurate [29].	Phenylalanine: Hippurate is a glycine conjugate of benzoate by liver or bacterial metabolism. Positive association with *ruminococcaceae clostridiaceae* [30].
Phenolsulfate	0.92× q = 0.0027		Phenylalanine and tyrosine:
o-cresol sulfate*	0.82× q = 0.0032	Changes in the cresol isomers are indicative of alterations in gut microbiome [31].	Conversion to 4-OH-phenylacetic acid by aerobic (enterobacteria) and anaerobic
3-methoxytyrosine	0.88× q = 0.00024	Metabolite in dopamine synthesis.	(clostridia) bacteria, before decarboxylation to p-cresol (putrefaction).
N-acetyl-cadaverine	0.89× q = 0.003	Polyamine - generated by metabolism of lysine by *E. coli*, *corynebacteria*, and *clostridia* [32].	Bacterial degradation of lysine

Footnotes: Metabolites that typically originate through bacterial metabolism of phenylalanine/tyrosine and lysine. x indicates fold change post vs. pre-antibiotic therapy. * VIP = 0.99.

increases in fat intake would be consistent with decreased energy expenditure and increased utilization.

Cholesterol was increased (1.36×, q = 0.002) post-therapy (Fig. 2). Despite high fat intake plasma/serum cholesterol levels are decreased in CF [40,41] with increased cholesterol oxidation compared to non-CF [42]. Therefore the increase in cholesterol post therapy seen here would be consistent with decreased catabolism of cholesterol with clinical improvement after therapy.

Phospholipids are essential to cell membrane stability and have important roles in cell signaling. Sphingolipids and ceramide metabolites were increased as a class post therapy except for the pro-inflammatory ceramide products sphingosine and sphingosine-1-phosphate (S-1-P), which were unchanged post therapy. This suggests not only increased lipid availability but changes in inflammation, specifically that a decrease in ceramidase activity is responsible for reduced pro-inflammatory S-1-P production driving ceramide to sphingomyelin through elevated sphingomyelin synthase activity (Fig. 3). If this switch from pro-inflammatory to anti-inflammatory lipid production is the consequence of antibiotic treatment directly or is due to a decreased bacterial load is unclear. The role of ceramide in CF lung disease is debated but several studies have shown low ceramide in plasma of adults with CF compared to healthy adults and an important role for sphingolipids in inflammation [43,44].

As indicated in Figs. 3, 12-HETE a leukotriene derived from amino acid metabolism decreased in many subjects (0.98×, q = 0.018), especially the ratio of 12-HETE to arachidonate was decreased at end compared to start of therapy (mean ± SEM: End: 1.26 ± 0.27 vs. Start 3.30 ± 0.86, p=<0.001). Since di-gamma-homolinolenate competes with arachidonic acid for lipoxygenase thus inhibiting production of eicosanoids, the findings are consistent with decreasing inflammation. The ratio of n6 to n3 omega fatty acids, an indicator of inflammation, decreased at completion of antibiotics, i.e. the ratio of arachidonate (AA) (20:4n-6) to docosapentaenoate (n3-DPA) (pre: 1.23 vs. post 0.91; 0.74-fold; p = 0.002), the ratio of dihomo-linoleate (20:2n6) to n3-DPA ratio (0.84-fold decrease, p = 0.005) and lower arachidonate/docosahexaenoate (DHA; 22:6n3) ratio (0.80-fold, p = 0.002).

The alterations in free fatty acids may indicate improved utilization, decreased inflammation (i.e. lower n6/n3 ratio) and decreased use of these metabolites for eicosanoid metabolism. Prior reports indicated elevations in the AA/DHA ratio in CF compared to non-CF subjects which were not associated with nutritional status [45], and cross-sectional studies within a CF cohort did not show differences by severity of lung disease [46].

Complex phospholipids as a class were increased post therapy, e.g. 1-palmitoyl-2-linoleoyl-GPC (16:0/18:2) or 1-stearoyl-2-linoleoyl-GPC (18:0/18:2) (1.25×, q = 0.0091 and 1.29, q = 0.0042, respectively), with increases in choline phosphate (1.49×, q = 0.049) as the precursor for choline, which itself was not statistically increased post-therapy (1.22×, q = 0.177). Altered phosphatidylcholine metabolism has been reported for CF compared to non-CF with some phosphatidylcholines being even lower in patients with severe vs. mild disease [47]. A correlation of phosphatidylcholine and choline to FEV_1 has also been reported in CF [48,49]. Such findings thus are consistent with increased phospholipids at end of therapy here.

3.5. Findings indicating decreased oxidative stress and inflammation post-antibiotic therapy

3.5.1. Histidine and purines

The essential amino-acid histidine (1.23×, q = 0.005) and the downstream metabolite N-acetyl-1-methylhistidine (2.11×, q = 0.013) were elevated at the end of therapy. In addition to its role in protein synthesis histidine and other imidazole compounds have anti-oxidant, anti-inflammatory and anti-secretory properties through the NFkB pathway [50]. Thus elevations at end of therapy may indicate decreased catabolism and decreased oxidative stress. Another indication for decreased oxidative stress at end of therapy was the 2.49 fold increase in retinol (q = 0.001) and retinal (2.18×, q = 0.01). In CF low vitamin A levels have been associated with

Table 3
Examples of metabolites indicating enhanced lipid availability/utilization.

Name of metabolite	Fold change post/pre (q value)	Pathway
2-linoleoylglycerol (18:2)	3.64 (0.009)	Monoacyl-glycerol
1-palmitoyl-2-linoleoyl-glycerol (16:0/18:2)	2.22 (0.010)	Diacyl-glycerol
laurate (12:0)	1.76 (0.036)	Medium Chain fatty acid
propionylcarnitine	1.72, (0.001)	Acyl carnitine
stearoylcarnitine	1.56 (0.005)	

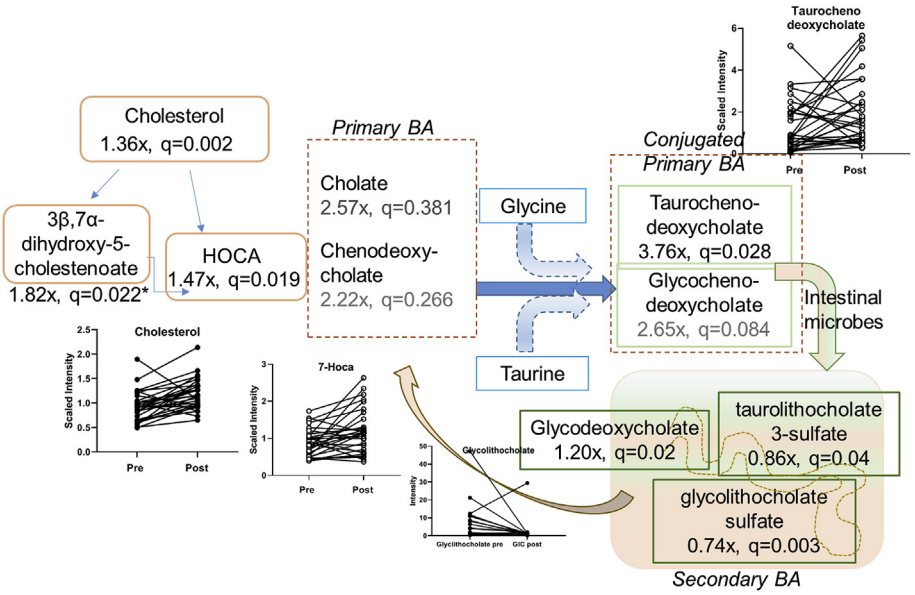

Fig. 2. Changes in bile acid (BA) precursors (e.g. 7-α-OH-3-oxo-cholestenoate (HOCA), and in primary and secondary bile acids indicate increased bile acid synthesis due to, or accompanied by decreases in intestinal BA re-circulation. Fold change indicated by x, with corresponding q value. Non-significant changes by *t*-test in lighter print.

more frequent exacerbations [51] and were negatively correlated to CRP [52]. Vitamin A intake increased significantly in the 17 subjects who had paired food-records, yet there was no correlation of vitamin A intake and retinol levels. These findings indicate the importance of inflammation rather than solely nutritional intake as a cause of lower vitamin A at time of exacerbation.

Previous metabonomic studies in CF found a trend for higher plasma hypoxanthine at baseline compared to exacerbation status without changes in purine levels [18]. In airway epithelial cultures, adenosine, inosine, hypoxanthine and guanosine, were decreased in CF compared to non-CF [53]. Although purines (inosine, guanosine, hypoxanthine or xanthine) were increased in some subjects there was no consistent increases after therapy in this study.

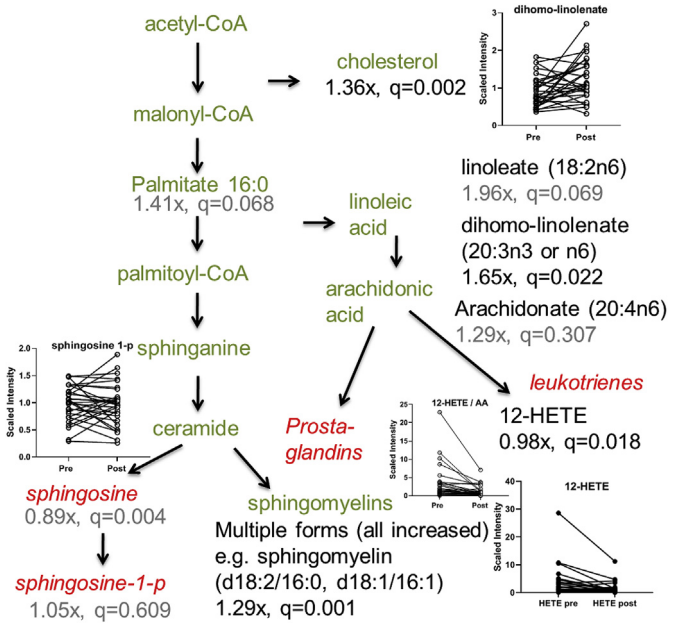

Fig. 3. Diagram of increased lipid synthesis pathway (green) and reduced production (red, italic) in response to antibiotic treatment in CF. Antibiotic treatment reduces pro-inflammatory lipid signaling. Fold change indicated by x, with corresponding q value. Non-significant changes in lighter print. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5.2. *Polyamines as markers of inflammation*

Polyamines (putrescine, spermidine, spermine, N1,N2-diacetylspermine) are polycationic amines with multiple functions in cell proliferation, angiogenesis and inflammation (Reviewed by Lenis and Hussain) [54,55]. Biosynthesis of polyamines results from conversion of arginine to ornithine, catalyzed by action of nitric oxide synthases and arginase (Fig. 4). Ornithine is the precursor for putrescine which is quickly metabolized into the polyamines spermidines and spermines. Post therapy levels of spermidine and N1,N2-diacetylspermine were lower compared to start of therapy without changes in either ornithine or arginine thus implicating decreases in the biosynthetic pathway of polyamines. The N-acetylated intermediates for arginine biosynthesis, N-delta-acetylornithine and N-acetylcitrulline were elevated at end of therapy.

These findings indicate increased arginine production (through increases in NOS and decreased activity of arginase and ODC) with higher availability into the NO pathway with resolution of exacerbation. This is consistent with prior reports of low NO production and L-arginine deficiency in CF airways [56]. Another study reported a reduction in sputum arginase levels with concomitant elevation of polyamines at times of CF exacerbations and compared to healthy subjects [57]. These findings in sputum are consistent with the lower serum spermine levels at resolution of exacerbation seen here. Changes in polyamines are not specific to CF as enhanced arginine synthesis/availability was seen in patients with COPD after addition of doxycycline as an anti-inflammatory agent [58]. Lower polyamine levels at resolution of CF exacerbation may be also related to lower cell proliferation rates and decreased oxidative stress as polyamines are elevated in diseases with increased cell turn-over e.g. cancer and in obese children where spermine levels correlated to markers of oxidative stress [59].

3.5.3. *Tryptophan/kynurenine pathway*

Tryptophan and several of its metabolites were elevated at the

end of therapy. Tryptophan metabolism involves microbial and host related reactions [60] and as sketched out in Fig. 5. Post-therapy tryptophan and its oxygen radical scavenging form, N-acetyl-tryptophan (1.84×, q = 0.036) were elevated with trends for higher xanthurenate (4.4 fold, q = 0.06) as metabolites in the kynurenine pathway.

Bacterial derived metabolites of tryptophan, e.g. methyl indole-3-acetate (3.06×, q = 0.15) and the short chain fatty acid indole-proprionate were elevated at end of therapy, with other short chain fatty acids e.g., indoleacetate and 3-indoxy-sulfate showing non-significant elevations. Serotonin and its metabolite 5-hydroxyindoleacetate were not changed after therapy.

The tryptophan-kynurenine pathway is catalyzed by indole-amine-2-3-dioxygenase (IDO) and kynurenine is the main metabolite of IDO. As a surrogate measure of IDO activity the kynurenine/tryptophan (Kyn/Trp) ratio can be used. IDO is induced by IFN-y and TNF-α and a higher Kyn/Trp ratio is a marker of increased inflammation (reviewed by Yeung) [60]. The Kyn/Trp ratio has been shown to be elevated in plasma of patients with COPD at time of exacerbation compared to resolution [61]. In plasma of patients with chronic enteropathy the Kyn/Trp ratio was positively correlated to IFN-y and higher tryptophan levels were predictive of improved growth in the subsequent months [62]. In our study, the Kyn/Trp ratio decreased after therapy i.e. 1.15 vs. 0.89 (p = 0.04) consistent with resolution of inflammation.

3.6. Limitations and comparison to literature

A limitation of this study is that metabonomics does not include absolute quantification of metabolites and as healthy control subjects were not included individual metabolites cannot be compared to normal values. However, sample collection and processing was identical to our prior study comparing CF and non-CF, which allows extrapolation of pathways that are altered in CF [14]. Several of our findings overlap with the changes in serum metabolites and pathways described 6-months after CFTR corrector therapy [17]. These included alterations in bacterial derived amino acid metabolites and bile acids. Interestingly, phospho- and sphingolipids were decreased after CFTR corrector therapy but increased in our study. Potential explanations include that recovery from exacerbation state reflects acute reversal of a catabolic state or that increased activity of CFTR may lead to increased insertion of these lipids into cell membranes as postulated by Kopp et al. [17].

Missing clinical data occurred due to unreliable results from spirometry and the fact that not all subjects completed food diaries at both time points, which may decrease the power of clinical data analyses. Yet, study subjects were homogeneous in regards to CFTR mutations, were old enough to perform spirometry, and had similar respiratory pathogens.

Metabonomics in CF respiratory secretions have shown markers of neutrophilic inflammation [63], alterations in lipid biomarkers, e.g. 12-HETE [16]. The serum metabonomics approach shows that markers of increased inflammation are also detectable systemically and additionally showed interesting changes in bacterial metabolites and bile-acids, likely linked to intestinal manifestations of antibiotic therapy and CF disease.

Two prior studies had conducted metabolomics profiling at time of CF pulmonary exacerbation using plasma samples [18,19]. There are several methodological aspects that do not allow full comparison of metabolites. Contrary to these other studies, all samples here were collected after overnight fasting to reduce dietary effects. We chose to use serum based on prior methodological aspects of metabolomics in healthy subjects that indicated less variance in peak for many metabolites, less effect of incubation/processing, and fewer effects from red blood cells in serum compared to plasma [64]. Sampling time points differed between studies: Alvarez et al., who focused on effects of high dose vitamin D supplementation during treatment of pulmonary exacerbation in adults with CF, obtained repeat samples already after one week of antibiotics [19]. Laguna et al. obtained the second sample at a clinic visit, i.e. the time interval was longer than in our study and time between the two samples varied between patients [18]. Although neither serum nor plasma can determine lung specific markers, blood based samples provide an assessment of changes in the whole body/organ

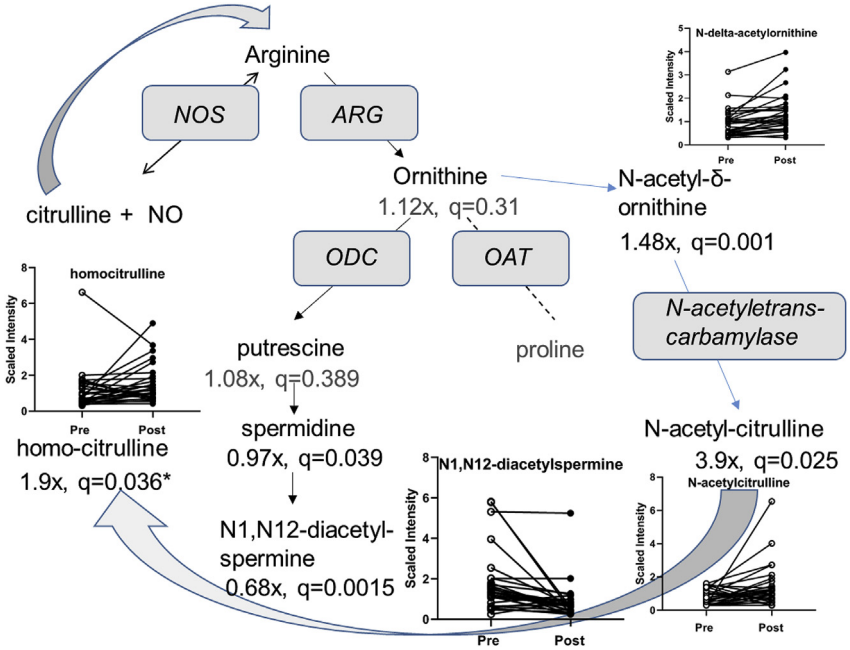

Fig. 4. Spermidine pathway showing decreases in polyamines post-therapy. Enzymes are indicated in italic font: *NOS* nitric oxide synthase; *ARG* arginase; *ODC* ornithine decarboxylase; *OAT* ornithine aminotransferease. Fold change in metabolites are indicated by x, with corresponding q value. Non-significant changes in lighter print.

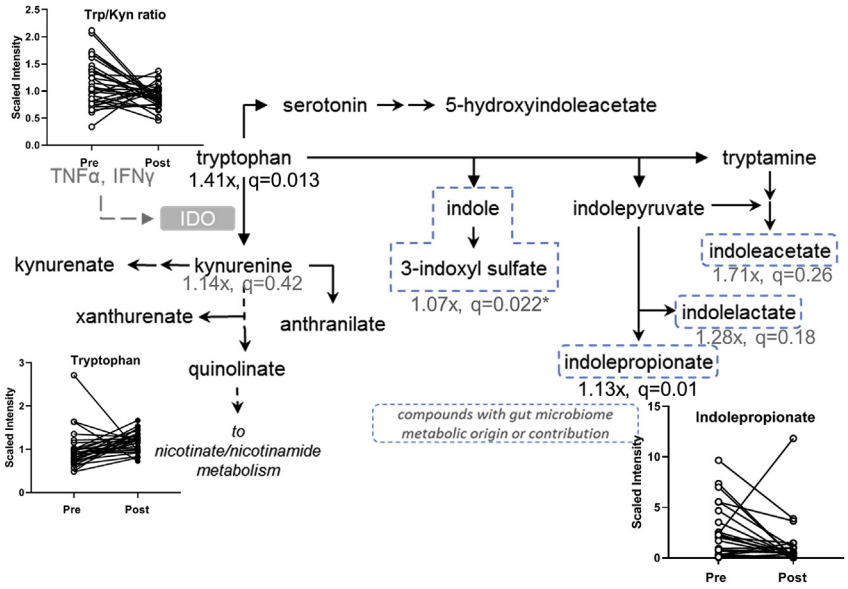

Fig. 5. Tryptophan and kynurenine pathway indicate decreases in inflammation. Indoleamine-2-3-dioxygenase (IDO) is the key step for metabolism of tryptophan along the kynurenine pathway. IDO is induced by IFN-y and TNF-α. Fold change in metabolites are indicated by x, with corresponding q value. Non-significant changes in lighter print.

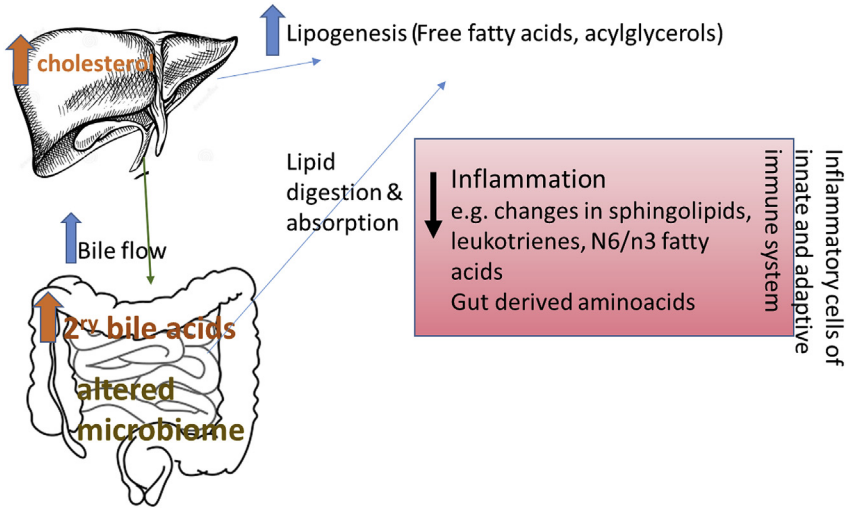

Fig. 6. Summary of serum metabolomics at resolution of exacerbation.

systems (Fig. 6).

In conclusion this global metabonomic profiling study showed that pulmonary exacerbations in CF are associated with changes that are measurable systemically. The key pathways affected were 1) Microbial derived amino acid metabolites presumably related to gut microbiome with concomitant increased bile acid production. 2) Enhanced substrate utilization indicated by increased lipid metabolites and β-oxidation, and 3) Decreases in oxidative stress (histidine-imidazoles) and inflammation (leukotriene, the arginine-polyamine, and the kynurenine-tryptophan pathways).

Statement of authorship

MSM, WS, JM designed the research project. MSM and research coordinators were responsible for subject recruitment, sample collection, and having metabolomics analyses done. BM performed food record analyses and interpretation. MSM and WS analyzed the data, MSM, JM, and TJK had primary responsibility for content. All authors read and approved the final manuscript.

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Coi statement

None of the authors has a conflict to disclose in regards to this work or manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metop.2019.100010.

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