

ARTICLE

Metabolomic profiling of extraesophageal reflux disease in children

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Abstract

Although respiratory symptoms in children are often attributed to gastroesophageal reflux disease, establishing a clear diagnosis of extraesophageal reflux disease (EERD) can be challenging, as there are no sensitive or specific EERD biomarkers. The aim of this study was to evaluate the metabolite profile in bronchoalveolar (BAL) fluid from children with suspected EERD and assess the impact of reflux treatment on these metabolites. In this prospective pilot study, we performed nontargeted global metabolomic profiling on BAL fluid from 43 children undergoing testing with bronchoscopy, upper endoscopy, and multichannel intraluminal impedance with pH (pH-MII) for evaluation of chronic respiratory symptoms. Twenty-three (54%) patients had an abnormal pH-MII study. Seventeen (40%) patients were on proton pump inhibitors (PPIs) for testing. Levels of histamine, malate, adenosine 5'-monophosphate, and ascorbate were significantly lower in subjects with abnormal pH-MII studies compared to those normal studies. Furthermore, in children off PPI therapy, those with abnormal pH-MII studies had robust increases in a number of glycerophospholipids within phospholipid metabolic pathways, including derivatives of glycerophosphorylcholine, glycerophosphoglycerol, and glycerophosphoinositol, compared to those with normal pH-MII studies. These findings offer insight into the impact of reflux and PPIs on the lungs and provide a foundation for future studies using targeted metabolomic analysis to identify potential biomarkers of EERD.

Study Highlights**WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?**

Establishing an association between gastroesophageal reflux disease and respiratory disease in children is challenging as there are no sensitive or specific biomarkers for extraesophageal reflux disease (EERD). Metabolomic analysis of bronchoalveolar lavage (BAL) fluid has offered promising insight into possible biomarkers for a variety of respiratory diseases but has never been studied in children with suspected EERD.

WHAT QUESTION DID THIS STUDY ADDRESS?

This study addressed the questions of (1) whether there are differences in BAL metabolites in children with abnormal reflux testing with multichannel intraluminal

impedance with pH and (2) whether reflux therapy with proton pump inhibitors was associated with different metabolite profiles.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

We found that there were significant increases in a number of glycerophospholipids within phospholipid metabolic pathways in the BAL of children with untreated reflux, suggesting that gastroesophageal reflux may impact the lung metabolome.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

These findings may provide mechanistic insight into reflux-induced lung injury and the impact of acid suppression medications on the lungs, which may help guide future biomarker discovery.

INTRODUCTION

Extraesophageal conditions, such as asthma, pneumonia, bronchiectasis, sinusitis, cough, and wheezing, are often attributed to gastroesophageal reflux disease (GERD).¹ However, establishing a clear pathophysiologic link between GERD and these respiratory symptoms can be challenging and patients often undergo costly evaluations to diagnose extraesophageal reflux disease (EERD).² As a result, there has been considerable interest in the identification of biomarkers that directly assess the impact of reflux on the lungs as a diagnostic approach to EERD. Although a few biomarkers, such as pepsin and the lipid-laden macrophage index, have been proposed, these have shown poor sensitivity and specificity when compared to the gold standard reflux testing and endoscopy.^{3–8} More recently, metabolomic analyses have shown promise in identifying biomarkers for the early diagnosis and characterization of a variety of respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis.^{9–11} Furthermore, metabolomic analysis may offer useful biomarkers of airway inflammation and lung injury in these patients and metabolites involved in inflammatory pathways have been linked to poorer outcomes in patients with respiratory disease.^{12–14} Although metabolomic studies have offered promising directions for biomarker discovery in other models of respiratory disease, no studies to date have investigated the lung metabolome in children or adults with suspected EERD.

In clinical practice, many patients with symptoms of EERD are prescribed empiric trials of acid suppression medications, such as proton pump inhibitors (PPIs). However, it is unclear whether this practice improves outcomes. In randomized controlled clinical trials, PPIs have not been shown to offer significant benefit in improving symptoms of chronic cough,¹⁵ asthma,¹⁶ or laryngitis.¹⁷ From a pathophysiologic standpoint, there is limited evidence on the effect of PPIs on the lungs. Some preclinical studies in murine

models of lung injury suggest that PPIs may reduce lung inflammation by suppressing expression of pro-inflammatory molecules and inhibiting progression of fibrosis when compared to controls,^{18,19} however, this has not been shown in humans. No studies have evaluated biomarker response to therapy in patients with suspected EERD.

The objectives of this study were to evaluate the metabolite profile in bronchoalveolar (BAL) fluid from children with suspected EERD and assess the impact of reflux treatment on these metabolites. We hypothesized that subjects with respiratory symptoms who have pathologic reflux measured by impedance testing have differences in BAL metabolites compared to subjects without pathologic reflux.

METHODS

Subjects

This was a prospective study to determine the inflammatory profiles in the lungs of children with and without GERD. In this study, we collected BAL fluid from children 1–18 years of age who underwent bronchoscopy, esophagogastroduodenoscopy (EGD) and multichannel intraluminal impedance with pH (pH-MII) testing at the discretion of their primary gastroenterologist and pulmonologist for evaluation of chronic respiratory symptoms between 2009 and 2014. All participants completed a baseline questionnaire highlighting gastrointestinal and respiratory symptoms, medical history, and medication use. Patients who reported stopping their PPI more than 48 hours prior to their procedures were considered “off PPI” and those who reported taking their PPI within 48 h of the procedure were considered “on PPI,” based on our institutional protocol. Criteria for on and off PPI were developed based on PPI pharmacokinetic studies and highlight the rapid metabolism of PPIs in pediatric patients.^{20–22} The decision to perform studies on or off PPI was at the discretion of the referring physician. Collection

of BAL fluid was performed during the bronchoscopy while patients were under general anesthesia through an endotracheal tube in distal airways of the right middle lung or the area of most visually inflamed lung.^{7,23,24} All samples were stored at -80°C . The study was approved by the Boston Children's Hospital Institutional Review Board, all research was conducted in concordance with the Declaration of Helsinki and informed consent was obtained from all participants or their guardians.

Multichannel intraluminal impedance with pH

In all patients, a pH-MII study was completed within 4 months of the EGD. Patients underwent pH-MII recording for a minimum of 18 h and ate at least three meals during this time. Meals were excluded from analysis. Studies were interpreted using standardized diagnostic criteria, first using Autoscan software (Sandhill Scientific) followed by confirmation with manual review by one of the study investigators (authors L.M. or R.R.). Acid reflux episodes were defined as retrograde bolus flow detected by at least two consecutive MII sensors and which were associated with a drop in pH to less than 4. Nonacid reflux episodes were defined as retrograde bolus flow detected by at least two consecutive MII sensors, which did not have any associated drop in pH to less than 4. Drops in pH to less than 4 without any associated reflux on MII were defined as pH only events.²⁵ The percentage of time that refluxate was present in the proximal esophagus was calculated by dividing the sum of the proximal bolus clearance times by the total study duration. Full column reflux events were defined as reflux events reaching the proximal two impedance sensors. The pH portion of the pH-MII study was considered abnormal if the pH was less than 4 for greater than or equal to 6% of the study.²⁶ The MII portion of the study was considered abnormal if there were greater than or equal to 73 reflux episodes.^{27,28} Patients were considered to have a normal reflux burden if both the pH and the MII portions of the study were normal.

Subject groups

Subjects were categorized into four groups. Patients with an abnormal pH-MII study (due to either an abnormal pH or an abnormal MII portion of the study) who were off PPI were designated as GER+PPI-. Those with an abnormal pH-MII study who were tested on PPI were categorized as GER+PPI+. Subjects with a normal pH-MII study who were off PPI were designated as GER-PPI- and those on PPI therapy for the study were designated as GER-PPI+.

Metabolomics analysis

Nontargeted global metabolomic profiling was performed through Metabolon (Durham, NC) on BAL samples collected during bronchoscopy. The sample processing and preparation techniques, mass spectrometer platforms, quality assurance components, and data analysis methods have been previously described in detail.²⁹ Briefly, samples were analyzed in concert with several well-characterized standards, which were designed to assess variability and verify performance of the extraction techniques and instrumentation. Ultrahigh performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) was performed using a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer (MS) interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for quality-control and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition. MS peaks were identified as previously described.²⁹ Peaks were quantified using area under the curve.

Statistical analysis

Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, statistical analyses were performed in ArrayStudio and SPSS version 24. Demographic and baseline characteristics were summarized using means (SDs) or frequencies (percentages). *T*-tests and χ^2 tests (or Fisher's exact test for cell counts <5) were used to compare continuous variables and categorical variables, respectively. If the distribution of a continuous variable was skewed, median (interquartile range) and Wilcoxon test were performed instead. Analysis of variance (ANOVA) was used to identify biochemicals that differed significantly between experimental groups. The false discovery rate (*q* value) was calculated to take into account multiple comparisons. Spearman correlations were used for relationships between percent neutrophils and metabolites. Pathway enrichment analyses were carried out using ANOVA to identify pathways with metabolites enriched at the top of the list of metabolites ranked by *p* value as principal components analysis did not differentiate global metabolic profiles in patients with reflux/no reflux. Fold changes were

calculated as the ratio of mean metabolite levels between the two groups.

RESULTS

Subjects

Metabolomic analysis was performed on BAL fluid from 43 children. The mean age was 8.7 ± 4.4 years and 44% of subjects were girls. Of these, 17 (40%) were on PPI (PPI+) at the time of the study, with a mean PPI dose of 1.0 ± 0.4 mg/kg/day. Twenty-six (60%) patients were off PPI at the time of the study (PPI-). Twenty-three (54%) patients had an abnormal pH-MII study (GER+) and 20 (46%) had a normal pH-MII study (GER-). Based on pH-MII study results and medication use, subjects were classified into one of the following groups: GER+PPI- ($n = 11$), GER+PPI+ ($n = 12$), GER-PPI- ($n = 15$), and GER-PPI+ ($n = 5$). Baseline demographic data among the four groups is presented in Table 1.

Endoscopy and bronchoscopy

Esophageal biopsies showed evidence of microscopic esophagitis, defined as greater than 0 eosinophils per high power field, in 17 (40%) subjects. There was no association

between a normal or abnormal pH-MII study and the presence or absence of esophagitis on biopsy ($p = 1.0$). There was no correlation between the percentage of neutrophils on BAL and the total number of reflux episodes ($r = -0.15$, $p = 0.36$), number of acid reflux episodes ($r = -0.07$, $p = 0.65$), number of full column reflux episodes ($r = -0.18$, $p = 0.26$), the percentage of total study time refluxate was present in the proximal esophagus ($r = -0.28$, $p = 0.07$), or the percentage of the study the pH less than 4 ($r = -0.15$, $p = 0.32$). Positive BAL bacterial cultures were found in 28% of patients. There was no association between a positive bacterial culture and any individual impedance parameter ($p > 0.32$).

Impedance testing

Symptoms reported during impedance testing included: cough (88%), vomiting (12%), reflux (9%), heartburn (5%), and gagging (5%). Impedance parameters by category are presented in Table 2. Only the percentage time of pH less than 4 differed significantly between groups.

Metabolomics analysis

Nontargeted metabolomics analysis of BAL samples from 43 subjects was performed. A total of 255 compounds of known identity were identified in our cohort. Using

	GER+PPI- <i>N</i> = 11	GER+PPI+ <i>N</i> = 12	GER- PPI- <i>N</i> = 15	GER- PPI+ <i>N</i> = 5
Demographics				
Age (years)	7.4 ± 4.5	10.2 ± 3.9	8.2 ± 3.7	9.7 ± 7.1
Female	7 (64)	4 (33)	8 (53)	0 (0)
Body mass index (kg/m ²)	18.7 ± 5.6	20.4 ± 5.9	18.9 ± 4.5	22.7 ± 5.7
Comorbid conditions				
Gastrostomy tube	3 (27)	0 (0)	0 (0)	0 (0)
Gastrojejunostomy tube	0 (0)	1 (8)	0 (0)	1 (20)
Asthma	9 (82)	10 (83)	12 (80)	4 (80)
Eczema	2 (18)	5 (42)	6 (40)	2 (40)
Seasonal allergies	3 (27)	3 (25)	10 (67)	2 (40)
Reflux	6 (55)	8 (67)	7 (47)	3 (60)
Dysphagia	5 (46)	4 (33)	2 (13)	3 (60)
Croup	6 (55)	6 (50)	8 (53)	1 (20)
Chronic cough	9 (82)	9 (75)	12 (80)	3 (60)
Sinus disease	3 (27)	7 (58)	8 (53)	1 (20)
Pneumonia	3 (27)	4 (33)	5 (33)	1 (20)

TABLE 1 Subject characteristics

Notes: Shown are mean \pm sd or *N* (%).

Abbreviations: GER, gastroesophageal reflux; PPI, proton pump inhibitor.

TABLE 2 Impedance parameters

	GER+PPI– <i>N</i> = 11	GER+PPI+ <i>N</i> = 12	GER– PPI– <i>N</i> = 15	GER– PPI+ <i>N</i> = 5	<i>p</i> value
Percentage time pH <4	11.2 ± 8.0	8.9 ± 6.9	3.1 ± 1.9	1.7 ± 1.7	0.001
No. acid episodes	33.3 ± 15.2	36.9 ± 34.2	19.9 ± 11.2	10.8 ± 13.8	0.06
No. nonacid episodes	25.3 ± 25.7	51.5 ± 82.4	14.6 ± 12.2	22.8 ± 20.7	0.24
No. pH only episodes	24.2 ± 16.5	17.3 ± 12.3	14.4 ± 13.8	8.4 ± 7.6	0.15
No. full column episodes	33.7 ± 22.8	53.1 ± 61.2	17.3 ± 13.7	17.4 ± 15.1	0.07
Total BCT (sec)	2532 ± 1854	4242 ± 8443	822 ± 474	898 ± 1029	0.26
Total proximal BCT (sec)	1210 ± 1070	4445 ± 12702	344 ± 252	509 ± 827	0.44
Percentage time refluxate present in the proximal esophagus	1.4 ± 1.2	5.6 ± 16.1	0.4 ± 0.3	0.7 ± 1.1	0.44
Cough reported during study	11 (100)	12 (100)	13 (87)	5 (100)	0.32
Symptom index for cough (%)	29.7 ± 12.8	51.9 ± 26.4	29 ± 20	31.3 ± 30.6	0.06

Notes: Shown are mean ± SD or *N* (%). The *p* value from analysis of variance.

Abbreviations: BCT, bolus clearance time; GER, gastroesophageal reflux; PPI, proton pump inhibitor.

TABLE 3 Phospholipids differing significantly between patients with normal and abnormal impedance studies in those tested off proton pump inhibitors

Metabolite	Fold change	<i>p</i> value	<i>Q</i> value
1-stearoyl–2-oleoyl-GPI	2.66	0.001	0.087
1,2-dipalmitoyl-GPC	3.42	0.001	0.087
1-stearoyl–2-oleoyl-GPG	3.84	0.001	0.087
1,2-dioleoyl-GPI	3.34	0.002	0.087
1-palmitoyl–2-palmitoleoyl-GPC	5.16	0.002	0.087
1-palmitoyl–2-oleoyl-GPC	3.85	0.003	0.087
1-palmitoyl–2-stearoyl-GPC	3.85	0.003	0.087
1-palmitoyl–2-alpha-linolenoyl-GPC	6.19	0.004	0.087

Notes: The *p* value from analysis of variance contrasts.

Abbreviations: GPC, glycerophosphorylcholine; GPG, glycerophosphoglycerol; GPI, glycerophosphoinositol.

two-way ANOVA contrasts, there were 4 metabolites which were significantly lower at the $p \leq 0.05$ level (histamine, malate, adenosine 5'-monophosphate, and ascorbate) in subjects with abnormal pH-MII studies (GER+PPI– and GER+PPI+) compared to those with normal pH-MII studies (GER–PPI– and GER–PPI+). There were no significant

differences in any metabolites for the main effect of PPI in those on (GER+PPI+ and GER–PPI+) and off (GER+PPI– and GER–PPI–) PPIs on ANOVA contrasts ($p > 0.05$). To evaluate the interaction between pH-MII results and PPI treatment, ANOVA contrasts were then sorted by *p* value to identify metabolic pathways enriched at the top of the list of significant metabolites.

Off PPI: Metabolites differentiating abnormal versus normal pH-MII studies

In subjects with abnormal pH-MII studies off PPI (GER+PPI–), pathway level subanalysis revealed a robust increase in many phospholipid classes, including derivatives of glycerophosphoethanolamine (GPE), glycerophosphorylcholine (GPC), glycerophosphoserine, glycerophosphoglycerol (GPG), and glycerophosphoinositol when compared to those with normal pH-MII studies off treatment (GER–PPI–; Table S1). After adjustment for multiple comparisons, 8 glycerophospholipids differed significantly among these groups, with a *q* value less than 0.10 (Table 3). There were no correlations among these eight metabolites and any individual impedance parameter ($p > 0.10$).

On PPI: Metabolites differentiating abnormal versus normal pH-MII studies

After adjustment for multiple comparisons, there were no significant differences in metabolite profiles between those with abnormal pH-MII studies (GER+PPI+) versus those with normal pH-MII studies (GER–PPI+) in PPI treated patients. There was a trend toward lower levels of the phospholipid metabolites GPC, GPE, phosphoethanolamine, and 1-palmitoleoyl-2-linoleoyl-GPC, and lower levels of the nucleotide metabolites adenosine and cytidine 5'-monophosphate, in the GER+PPI+ group when compared to the GER–PPI+ group, although these did not reach statistical significance after adjustment for multiple comparisons (Table S1). There were no correlations between these metabolites and any individual impedance parameter ($p > 0.35$). Adenosine was negatively correlated with the percentage of neutrophils on BAL ($r = -0.346$, $p = 0.023$).

Normal reflux burden: Metabolites differentiating PPI treatment versus no PPI treatment

In subjects with a normal reflux burden on pH-MII testing, those tested on PPI treatment (GER–PPI+) had a trend toward increased levels of phospholipid derivatives of GPC, GPG, and GPE in BAL when compared to those tested off PPI treatment (GER–PPI–), although these did not reach statistical significance after adjustment for multiple comparisons (Table S1).

Abnormal reflux burden: Metabolites differentiating PPI treatment versus no PPI treatment

In subjects with abnormal pH-MII studies, patients taking PPIs (GER+PPI+) had a trend toward lower levels of the phospholipid metabolite 1-palmitoyl-2- α -linolenoyl-GPC and lower levels of a number of carbohydrate metabolites compared to those tested off PPIs (GER+PPI–), although these were not significant after adjustment for multiple comparisons (Table S1).

DISCUSSION

In this novel prospective study, we used global metabolic profiling to characterize the metabolite profile in BAL fluid in children with chronic respiratory symptoms and suspected EERD. Our findings are most notable for significant increases in a number of glycerophospholipids within phospholipid

metabolic pathways in the BAL of children with untreated reflux. Furthermore, we found that PPI treatment may also play a role in altering metabolic patterns, with a trend toward lower levels of phospholipid metabolites in subjects with abnormal pH-MII studies on PPI therapy, and increases in phospholipids in subjects with normal pH-MII studies on PPI therapy. Our results form the basis for intriguing directions for future targeted metabolomic studies to discover potential biomarkers of EERD and could ultimately help guide future EERD diagnostics and therapeutics.

Glycerophospholipids play key roles in cellular function including membrane stability and fluidity, signal transduction, and vesicle trafficking.^{30,31} Within the lungs in particular, the glycerophospholipids observed to be elevated in patients with untreated reflux in our study are key intermediates in the formation of pulmonary surfactant and also feed into inflammation pathways via prostaglandin synthesis. Alterations in these phospholipids have been associated with impairment in the surface tension-lowering properties of surfactant, modulation of inflammatory responses, and modification of the host response to pathogens.³² In both preclinical and clinical studies, altered glycerophospholipid metabolites have been implicated in lung injury and inflammation in a number of pulmonary diseases, including lower respiratory tract infections, COPD, acute respiratory distress syndrome, bronchiolitis obliterans, and toxin-mediated lung injury.^{33–38}

Although a few prior studies have investigated select metabolite biomarkers of EERD in lung fluid, these studies have several limitations. Neujahr et al. performed metabolomic profiling in patients who underwent a lung transplantation with and without reflux and subsequent aspiration of gastric contents, defined by the presence or absence of bile acids in BAL fluid.¹⁴ The authors found that a number of metabolites differed between groups, particularly within pathways associated with microbial metabolism and metabolites previously reported to be associated with lung injury, such as T cell granzyme B level and chemoattractants CXCL9 and CXCL10. However, these findings are limited in their applicability to EERD, as bile acids have been shown to have limited sensitivity and specificity for diagnosing GERD when compared to traditional pH-metry and poor correlation with symptoms.^{39,40} In another study, Soyer et al. compared metabolites associated with oxidative stress (nitric oxide metabolites and total sulphhydryle) in the exhaled breath condensate (EBC) of children with chronic respiratory and gastrointestinal symptoms with and without reflux as defined by 24-h pH probe.⁴¹ However, the correlation between EBC and BAL metabolites is not clear and, furthermore, traditional pH-metry can underestimate the amount of reflux events by 50% or more when compared to pH-MII, limiting the generalizability of the study.^{25,42,43} Recently, the same group also studied a limited number of metabolites associated with oxidative stress (glutathione, 8-isoprostane, and cysteinyl-leukotriene)

in the EBC of patients with esophageal atresia and suspected reflux based on reported symptoms.⁴¹ Although the authors found a decrease in cysteinyl-leukotriene, a mediator of inflammation, in subjects treated with PPIs and with prior fundoplication, no objective assessments of reflux burden were made and, given the underlying dysmotility in this patient population, metabolite changes could relate to effects from esophageal stasis rather than reflux alone. We add to the existing literature by using pH-MII to objectively quantify reflux and using global metabolite profiling to identify broad areas of interest for additional targeted investigation.

Studies on the association between reflux and phospholipid metabolites have yielded conflicting results. Chang et al. did not observe any differences in dipalmitoylphosphatidylcholine (DPPC) concentrations, a main component of surfactant, in the BAL of children with reflux defined based on the presence of reflux esophagitis.⁴⁴ In contrast, in a -ell characterized group of adult patients who underwent lung transplantation and underwent esophageal manometry, 24-h ambulatory pH testing and nuclear medicine gastric emptying studies, D'Ovidio et al. found that the presence of reflux was associated with altered surfactant phospholipids, such as DPPC, phosphatidylglycerol, and sphingomyelin in BAL.⁴⁵ In addition to lipid mediators, Griese et al. also reported reduced levels of surfactant proteins SP-A and SP-D, proteins known to play key roles in host defense against pathogens, in children with reflux.⁴⁶

Evidence supporting the association between PPIs and metabolic alterations in the lungs is even more limited. Recent studies have found PPIs to have effects in the lungs beyond their role in gastric acid inhibition. In animal studies, omeprazole has been shown to attenuate hyperoxia-induced lung injury and increase surfactant proteins compared to controls.⁴⁷ In cell culture studies, esomeprazole has also been shown to have a protective effect on the lungs through suppression of pro-inflammatory molecules and antifibrotic mechanisms, findings that have also been replicated in mouse models of acute lung injury.¹⁸ However, no studies to date have investigated the relationship between PPIs and phospholipids in the human lungs.

Although our results are promising to guide future research, our study does have some limitations. First, to determine reflux burden, we used pH-MII testing to diagnose reflux. Whereas this diagnostic test only directly measures esophageal reflux and thus may not necessarily reflect the burden of extraesophageal reflux, pH-MII testing is considered the gold standard diagnostic test for GERD in both adult and pediatric consensus guidelines.^{28,48} Furthermore, in pediatrics, when nonacid reflux makes up 45–89% of all reflux episodes, pH-MII testing is the only method to reliably measure both full column and nonacid reflux.⁴⁹ Second, the sample size for this pilot study is small, and thus we may

have been underpowered to detect significant alterations in metabolite levels between sample groups. Although we found trends in altered metabolites between groups, such as increased phospholipid metabolites in subjects with normal pH-MII studies treated with PPIs and lower phospholipid levels in children with abnormal pH-MII studies treated with PPIs, these did not reach statistical significance after adjustment for multiple comparisons. A larger sample size will be needed to better understand the role of PPIs in this population of patients. Furthermore, global metabolomic profiles were assessed in this unsupervised discovery cohort. Our findings, while interesting to generate hypotheses and guide future studies, will need to be validated in a larger cohort and confirmed with targeted metabolomics studies. Finally, whereas we have a cohort of patients who were treated and a second cohort of patients who were not treated with PPIs, we do not have BAL samples in the same patients before and after starting PPIs. This would be an important next study to validate the results in this small series.

In conclusion, in this pilot study of children with suspected EERD, we found that BAL fluid from children with untreated reflux was associated with robust increases in a variety of glycerophospholipids when compared to those without reflux and off treatment. Treatment with PPI may also alter metabolite profiles. These findings may provide mechanistic insight into reflux-induced lung injury and the impact of acid suppression medications on the lungs, which may help guide future biomarker discovery.

CONFLICT OF INTEREST

All authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

L.B.M., C.R.E., and R.R. wrote the manuscript. L.B.M., C.R.E., and R.R. designed the research. L.B.M., K.M., and R.R. performed the research. L.B.M., C.R.E., and R.R. analyzed the data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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