Prevalence of Pediatric Aspiration-Associated Extraesophageal Reflux Disease

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IMPORTANCE The role of aspiration-associated extraesophageal reflux disease (AERD) in patients with chronic respiratory symptoms is not well defined. Identifying the frequency of AERD in these patients may provide guidance in their treatment.

OBJECTIVE To determine the prevalence of AERD in patients with chronic respiratory symptoms and to assess the utility of pepsin as a new marker for AERD.

DESIGN Case-control study performed from 2008 through 2012. Western blot analysis for pepsin and oil red O staining for lipid-laden macrophages (LLMs) was performed on bronchoalveolar lavage fluid specimens.

SETTING Tertiary referral center.

PARTICIPANTS Sixty-five patients (aged 4.5 months to 24 years) with chronic pulmonary disease, with or without tracheostomy, were compared with controls undergoing elective surgery who had no history of pulmonary disease.

MAIN OUTCOMES AND MEASURES Presence of pepsin and LLMs and quantity of LLMs in specimens.

RESULTS Seventy-six total patients participated: 34 patients who underwent bronchoscopy, 31 patients with tracheostomy, and 11 controls. Pepsin-positive bronchoalveolar lavage fluid specimens were identified in 25 patients who underwent bronchoscopy (74%) and 22 patients with tracheostomy (71%). All specimens from controls were negative for pepsin. Presence of LLMs was identified in specimens from 31 patients in the bronchoscopy group (91%), 16 patients in the tracheostomy group (52%), and 7 controls (64%), with a similar distribution of the quantity of LLMs in each lavage fluid specimen among the groups.

CONCLUSIONS AND RELEVANCE Patients with chronic pulmonary disease have a high prevalence of AERD, which may have important treatment implications. The presence of pepsin was a better predictor of AERD in patients with respiratory symptoms compared with controls than presence of LLMs. Detection of pepsin in bronchoalveolar lavage fluid specimens can serve as a biomarker for AERD and is potentially superior to the current method of measuring LLMs. Whereas there is a significant association between AERD and the presence of chronic respiratory symptoms, this study does not verify causation. Additional study investigating the mechanism of pepsin on the respiratory epithelium may further our understanding of the pathophysiologic characteristics of this association and provide additional management options for these patients.

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Direct aspiration of ingested material and reflux aspiration have both been implicated in the development and/or progression of pulmonary disease.\textsuperscript{1,2} Distinguishing between these 2 types of aspiration is important in guiding treatment recommendations. However, making a diagnosis of aspiration-associated extraesophageal reflux disease (AERD) continues to be difficult, as does defining its role in patients with chronic pulmonary symptoms. Clinical tests currently used to assess the presence of AERD have limitations. Modified barium swallow studies often have a poor negative predictive value, frequently missing episodes of reflux and intermittent aspiration.\textsuperscript{3} The use of 24-hour pH probe monitoring was considered the gold standard for diagnosing gastroesophageal reflux disease; however, this method may miss episodes of nonacidic reflux. Multichannel intraluminal impedance monitoring was introduced to help capture weakly acidic episodes of reflux.\textsuperscript{2-4} Prior investigators also looked at measuring glucose in tracheal secretions as a measure of aspiration without effective results.\textsuperscript{5} Measurement of lipid-laden alveolar macrophages (LLMs) from bronchoalveolar lavage (BAL) fluid specimens is the most widely used test to identify AERD. This test is based on the hypothesis that re-fluxate will be phagocytosed by alveolar macrophages and that staining for these in the BAL fluid would verify the presence of AERD.\textsuperscript{6} Prior studies have demonstrated conflicting results. Higher concentrations of LLMs in BAL samples were found in patients with lung disease and gastroesophageal reflux (GER).\textsuperscript{6} However, LLMs were also found in patients without GER and in control patients and thus are not great predictors of aspiration.\textsuperscript{1-7} Different methods of measuring the LLMs have been investigated, including the LLM index or classifying the amount of lipid in each cell. However, the diagnostic utility of these methods is limited and has shown variable results among studies.\textsuperscript{3,8,9}

Pepsin, an exogenous protein, has been proposed as a good biomarker of aspiration on the basis of the results of animal studies.\textsuperscript{10} Pepsin has been shown to potentially play a role in acute exacerbations of idiopathic pulmonary fibrosis\textsuperscript{11} and has been detected in patients requiring mechanical ventilation who are at risk for aspiration.\textsuperscript{2} Stovold et al\textsuperscript{12} used pepsin as a biomarker of gastric aspiration and reported elevated levels of pepsin in BAL fluid obtained from lung allografts, the highest levels being found in patients with acute rejection. Fischella et al\textsuperscript{13} also used pepsin as a biomarker for aspiration and reported that laparoscopic antireflux surgery is an effective means to prevent aspiration as defined by the absence of pepsin in the BAL fluid. Whereas research teams continue to produce studies that demonstrate the effective use of pepsin as a biomarker of aspiration, this technique has not been fully translated to the clinical setting, and often pathology laboratories are not fully equipped to perform this testing.

The purpose of this study was to determine the prevalence of AERD in our cohort of patients with chronic respiratory symptoms and in patients with tracheostomies by detecting the presence or absence of pepsin in BAL specimens. In addition, the effectiveness of pepsin as a biomarker for AERD was investigated by comparing the results of pepsin detection in the BAL specimens with the data measuring LLMs obtained from the same tracheal aspirate specimens. The findings from these study objectives may highlight the importance of more routine testing for pepsin in BAL specimens in specific patient populations.

Methods

Patient Selection and Study Design

The institutional review board at Children’s Hospital of Wisconsin approved this study (protocol 122706). Written informed consent was obtained for each enrolled patient. Participants were aged 4.5 months to 24 years and fulfilled 1 of the following criteria during the 4-year study period (February 2008 through June 2012): patient without pulmonary disease undergoing an elective procedure, patient undergoing a diagnostic bronchoscopy, or patient with a tracheostomy. Patients without a history of respiratory symptoms or GER undergoing an elective procedure were recruited to the control group. Patients with a history of chronic cough, wheezing, recurrent pneumonia, abnormal lung examination results, or increased work of breathing that warranted a diagnostic bronchoscopy were recruited to the bronchoscopy group. Patients with a previously diagnosed chronic lung disease with worsening symptoms requiring a diagnostic bronchoscopy were also enrolled in the bronchoscopy group. Patients with tracheostomy dependence requiring an airway evaluation were included in the tracheostomy group. In addition, there were 2 patients used as a control for the pepsin test who had laryngotraceal separation and tracheal gastric separation. Patient demographic data and clinical characteristics were collected by means of medical record review. Patients were excluded from the study if they lacked an adequate fluid specimen for complete analysis or were undergoing an elective procedure but had pulmonary disease.

Bronchoalveolar lavage fluid specimens were obtained by means of flexible bronchoscopy under general anesthesia from each participant in the bronchoscopy and tracheostomy groups. Lavage fluid specimens were also obtained from control patients during an unrelated surgical procedure. After intubation, 1 mL of normal saline was infused through the endotracheal tube and immediately suctioned. For participants who underwent a diagnostic bronchoscopy, a portion of the aspirated fluid collected during the procedure was used. Each specimen was assigned a code to correlate with the patient’s clinical data. Immediately after the specimen was obtained, it was placed on ice and transported to the research laboratory. Each specimen was mixed and divided into 2 samples. One sample was sent to a pathologist for additional LLM analysis, and the other was snap-frozen on dry ice and stored at –80°C for Western blot analysis.

Western Blot Analysis for Pepsin

An aliquot of 20 to 30 μL of lavage fluid was separated on a 10% sodium dodecyl sulfate polyacrylamide gel by means of electrophoresis. Purified human pepsin 3b (previously isolated from human gastric juice by means of ion exchange chromatography; Medical College of Wisconsin protocol
PRO0000475914 and human pepsinogen I (Sigma) were run alongside clinical samples as positive and negative controls, respectively. Protein was then transferred to a polyvinylidene difluoride membrane (GE Healthcare). Blots were incubated with rabbit anti-human pepsin HU3 peptide antibody (1:350 dilution)15 and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase diluted 1:5000 (Dako). All antibodies were diluted in 5% nonfat dried milk in phosphate-buffered saline with 0.1% polysorbate 20. Blots were exposed to enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Inc) followed by radiographic exposure and development. The presence or absence of a pepsin band was recorded. The person performing the Western blot for pepsin was not aware of the clinical findings.

Results

A total of 76 patients meeting the inclusion criteria were enrolled in the study during the 4-year study period. Demographic characteristics of enrolled patients are summarized in Table 1. Sixty-one percent were male and 39% were female, and the mean (range) age was 6.5 years (4.5 months to 24 years). In the tracheostomy group, 2 patients older than 18 years were included in the study because they were treated in our pediatric care center and had congenital disease processes that contributed to the long-term tracheostomy dependence and development of chronic lung disease. Of the 76 participants, 34 patients underwent bronchoscopy, 31 patients had a tracheostomy, and 11 were controls. There was no significant age difference between the control group and the 2 study groups (P = .99). Sex was not perfectly balanced between the bronchoscopy group and the tracheostomy group; however, this difference was not statistically significant (P = .34). Nine patients in the control group underwent elective procedures, including liver biopsy, osteotomy, toe amputation, implantation of osseointegrated bone-anchored hearing device, neck mass excision, incision and drainage of preauricular cyst, and palatal reconstruction. In addition, there was 1 patient who had a laryngotracheal separation and 1 patient with a history of a tracheoesophageal fistula with esophageal atresia after closure of fistula. These patients were also used as controls because they did not have a communication between the trachea and stomach to allow reflux of gastric contents.

In the bronchoscopy group, 16 patients had chronic respiratory symptoms, including recurrent wheezing, chronic cough, or shortness of breath and underwent a bronchoscopy for an airway evaluation; 10 patients had recurrent pneumonia; 1 patient had noisy breathing; 1 patient had regurgitation with concern for aspiration; 2 patients had abnormal lung examination results; and 1 patient had hemoptysis. One patient did not have a reason recorded. Two patients with a history of bilateral lung transplant were included because they were undergoing a diagnostic bronchoscopy for continued surveillance of the transplant. In the tracheostomy group, 15 patients had a tracheostomy performed for an anatomic abnormality, including bilateral vocal cord paralysis, upper airway obstruction, subglottic stenosis, and facial trauma; 9 patients had tracheostomy for neuromuscular disease; and 7 patients had tracheostomy performed for other reasons including chronic lung disease, central hypoventilation syndrome, hypoxic ischemic encephalopathy, upper airway obstruction accompanied by pulmonary hemorrhage, and upper airway obstruction accompanied by apnea.

Analysis of Diagnostic Bronchoscopy Specimen

Specimens obtained during diagnostic bronchoscopy were transported to the clinical laboratory at Children’s Hospital of Wisconsin, where the pathologist determined the presence of LLMs in each. The specimens were centrifuged, and cell suspensions from a portion of the bronchoscopy sample were prepared. These were stained with oil red O stain. Under light microscopy, 1 pathologist counted the number of LLMs in each specimen. The LLMs for each patient sample were additionally quantified on a 5-point scale as described by Corwin and Irwin.16 The LLM index was also determined by combining the scores for 100 consecutive macrophages. The pathologist performing this analysis was not aware of the clinical findings of the patient or the results of the pepsin analysis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (N = 76)</th>
<th>Control Group (n = 11)</th>
<th>Bronchoscopy Group (n = 34)</th>
<th>Tracheostomy Group (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>46 (61)</td>
<td>6 (55)</td>
<td>18 (53)</td>
<td>22 (71)</td>
</tr>
<tr>
<td>Female</td>
<td>30 (39)</td>
<td>5 (45)</td>
<td>16 (47)</td>
<td>9 (29)</td>
</tr>
<tr>
<td>Age, mean, y</td>
<td>6.5</td>
<td>6.2</td>
<td>6.5</td>
<td>6.6</td>
</tr>
<tr>
<td>BMI, mean, percentile</td>
<td>63</td>
<td>63</td>
<td>54</td>
<td>75</td>
</tr>
</tbody>
</table>

Abbreviation: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared).
tors (12 of whom had pepsin-positive BAL fluid samples). The majority of patients in both groups were using bronchodilators for control of respiratory symptoms. Administration of steroids was also evaluated in the bronchoscopy group. Of the 29 patients for whom steroid use data was available, 25 (86%) were receiving steroids, including 22 receiving inhalants only, 2 receiving both inhaled and oral steroids, and 1 receiving only oral steroids. All 3 patients receiving oral steroids had a pepsin-positive BAL specimen. Samples from patients who underwent Nissen fundoplication were positive for pepsin in the 1 patient in the bronchoscopy group who had the procedure and in 5 of 8 patients in the tracheostomy group. Few patients underwent a documented swallow study; these demonstrated aspiration in 1 of 5 patients in the bronchoscopy group and 3 of 18 patients in the tracheostomy group.

Western blot analysis for pepsin was performed on a BAL fluid sample from each patient. Pepsin was not detected in any of the samples from controls. Pepsin was detected in samples from 25 patients in the bronchoscopy group and 22 patients in the tracheostomy group. Data on LLMs were also collected. Lipid-laden macrophages were found in the BAL fluid samples from 7 patients in the control group, 31 patients in the bronchoscopy group, and 16 patients in the tracheostomy group. The results of Western blot and LLM testing are given in Table 3.

Receiver operating characteristic (ROC) analysis predicting patient group (control vs bronchoscopy group or tracheostomy group) from LLM data was performed. An examination of the specimens for the presence of any LLMs or pepsin revealed a moderate agreement between the presence of LLMs and pepsin for the bronchoscopy and tracheostomy groups (k = 0.3; P = .03) but no apparent agreement among controls (k = −0.2; P = .15). Examination of their ability to predict control vs at-risk patients revealed that both LLM and pepsin testing have reasonable area under the ROC curve, 0.53 and 0.86, respectively, which are not significantly different from each other. However, when LLM data were quantified as the number of LLMs among all macrophages in the specimen, there was no agreement between LLM and pepsin, and LLMs were not predictive of patients undergoing bronchoscopy or with tracheostomy. In addition, LLM data were not predictive of pepsin status when this quantification was performed (Figure).

### Discussion

Aspiration-associated extraesophageal reflux disease may have implications on our understanding and approach to patients with chronic respiratory symptoms and those with chronic lung disease. Using pepsin as a marker for AERD demonstrated a high prevalence of AERD in our cohort of patients with chronic respiratory symptoms and tracheostomy, with more than 70% of the patients in the 2 groups having pepsin detected in the BAL specimen. Our data suggest that the frequency of AERD is likely underestimated in patients with disease patterns similar to our study cohort, and it may play a stronger role in chronic pulmonary disease than previously recognized. Prior studies have begun to investigate the role of “silent” aspiration in some patient groups. Gopalarreddy et al identified a high rate of silent aspiration in critically ill pediatric patients requiring mechanical ventilation in the intensive care unit. Additionally, Krishnan et al demonstrated a high correlation between patients with respiratory disease and GER and the presence of pepsin in tracheal secretions. However, with use of conventional methods, detection of silent aspiration has been difficult and warrants the development of new techniques.

Previously, quantification of LLMs in a BAL specimen has been proposed as a measurement tool for detecting aspiration. Varying and conflicting data regarding the reliability of this test have been reported in the literature. The LLMs are not necessarily exogenous and may reflect the presence of phospholipid degradation from pulmonary inflammation or infection. In addition, a previous study demonstrated the presence of LLMs in control patients, further emphasizing the nonspecific nature of this marker. Although additional study is needed to confirm this, our findings suggest that LLM analysis has substantial potential to produce false-positive test results. Our data also suggest that analysis of LLMs misses some patients who truly have AERD. As such, the positive predictive value [true positive/(true positive + false positive)] of the LLM test seems poor.

### Table 2. Clinical Characteristics in Each Group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Bronchoscopy Group (n = 34)</th>
<th>Tracheostomy Group (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapy received, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPI</td>
<td>12 (35)</td>
<td>14 (45)</td>
</tr>
<tr>
<td>H2 blocker</td>
<td>5 (15)</td>
<td>5 (16)</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>3 (9)</td>
<td>0</td>
</tr>
<tr>
<td>Bronchodilators</td>
<td>26 (76)</td>
<td>26 (84)</td>
</tr>
<tr>
<td>Swallow study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Performed, No. (%)</td>
<td>5 (15)</td>
<td>18 (58)</td>
</tr>
<tr>
<td>Results positive for aspiration, No.</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviation: PPI, proton pump inhibitor.

### Table 3. Pepsin and Lipid-Laden Microphage (LLM) Results From Bronchoalveolar Lavage Fluid Specimens for Each Group

<table>
<thead>
<tr>
<th>Positive Results</th>
<th>Total (N = 76)</th>
<th>Control Group (n = 11)</th>
<th>Bronchoscopy Group (n = 34)</th>
<th>Tracheostomy Group (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin</td>
<td>47 (62)</td>
<td>0 (0)</td>
<td>25 (74)</td>
<td>22 (71)</td>
</tr>
<tr>
<td>LLMs</td>
<td>54 (71)</td>
<td>7 (64)</td>
<td>31 (91)</td>
<td>16 (52)</td>
</tr>
</tbody>
</table>
Conversely, pepsin detection is shown to be a superior test; pepsin is purely exogenous, unlike LLMs. In this study, all controls had negative results on this test, which is consistent with our knowledge of pepsin in physiologic conditions. Pepsin is only produced in the stomach by gastric chief cells, and thus its presence in BAL fluid indicates extraesophageal reflux and subsequent aspiration. Our anti–human pepsin can be used to discriminate between pepsin and pepsinogen, allowing pepsin to be used reliably to detect extraesophageal reflux and/or aspiration. Fisichella and colleagues also noted similar results in a prior study while evaluating the effectiveness of laparoscopic antireflux surgery in lung transplant recipients by measuring the presence of pepsin in BAL fluid specimens. These results were compared with BAL fluid specimens from 11 healthy control patients; all specimens from the control patients were negative for the presence of pepsin. Prior studies have also supported the use of pepsin testing as a measurement of aspiration.

Farrell et al looked at a group of patients with a proven macroscopic aspiration event who had significantly higher levels of pepsin compared with controls. In addition, a significantly higher proportion of patients with proximal GER had pepsin-positive samples and cough-related symptoms, thus supporting a mechanism of disease involving the negative impact of exposure of the respiratory epithelium to refluxate.

The suggested high prevalence of AERD in this study cohort has the potential to enable important management decisions to be made for these patients. Prior investigations of the effects of reflux on hypopharyngeal and laryngeal structures have shown that pepsin may damage the laryngeal epithelium. Pepsin, originating from laryngopharyngeal reflux, was shown to have a negative impact on the defense mechanisms of the laryngeal epithelium, including decreased levels of laryngeal carbonic anhydrase III and Sep70 protective proteins. There may be additional damage occurring in the respiratory epithelium as well. Animal studies have also shown pepsin to have an effect on cytokine expression and airway remodeling.

The frequency of silent aspiration in our patients with chronic respiratory symptoms may have additional implications on diagnosis and treatment options. Only half of the patients were receiving any form of antireflux therapy, and those who were receiving medications nevertheless had pepsin detected in the BAL specimen. Pepsin at neutral pH has been shown to retain its original activity and ability to be reactivated with drop in pH during a repeated reflux event or when taken up into an acidic intracellular environment.

Silent aspiration in these patients may contribute to worsening pulmonary function, and understanding its impact has the potential to guide change in both medical and surgical interventions for these often medically fragile patients.

In addition, it was interesting to look at the pepsin results of our subpopulation of patients who had previously undergone a Nissen fundoplication. It was surprising that 6 of these 9 patients had pepsin detected in the lavage fluid specimens despite the presence of the Nissen fundoplication. In-depth review of the medical records of these 6 patients revealed that 4 experienced recurrence of reflux symptoms after the fundoplication and were prescribed continuing proton pump inhibitor therapy. However, this finding is supported by several studies in the literature reporting high failure rates for Nissen fundoplication (60%-70%). In a large controlled study, Spechler et al found that 62% of adults were taking proton pump inhibitor medications for reflux symptoms at a 7-year follow-up after antireflux surgery. Postoperative outcome measurements are also not always objective and consistent; therefore, determining the success of surgery is sometimes difficult, and few studies have been performed.

Although our results demonstrate a high prevalence of pepsin-positive samples, we were unable to identify any specific clinical factors that may predict which patients are more likely to have silent aspiration events. In the absence of any specific factors, pepsin testing of BAL fluid samples—which seems to have a high positive predictive value—may be a feasible means to identify these patients.

There are several limitations to our study. First, the technique used to determine the presence of pepsin in the BAL specimens could not be used to quantify the amount of pepsin present. Therefore, the severity of the aspiration-associated reflux cannot be more precisely defined by this test. The amount of reflux necessary to cause lung disease is not understood. In addition, it is not known how long pepsin remains detectable in the BAL fluid, so there may be some false-negative test results in patients with intermittent AERD.

In conclusion, there is a high prevalence of AERD in pediatric patients with chronic respiratory symptoms and tracheostomy. Clinically, the impact of this finding is likely underestimated. Pepsin is a reliable biomarker to detect AERD and is practical to test for in the clinical setting. Using this test may allow improved recognition of AERD and lead to more focused management.
Prevalence of Pediatric AERD

ARTICLE INFORMATION

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Author Contributions: Drs Kelly, Parakininkas, Werlin, Southern, and Kerschner had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Parakininkas, Werlin, Johnston, Kerschner.

Acquisition of data: Kelly, Parakininkas, Southern, Johnston, Kerschner.

Analysis and interpretation of data: Kelly, Southern, Johnston, Kerschner.

Drafting of the manuscript: Kelly, Parakininkas, Southern, Johnston, Kerschner.

Critical revision of the manuscript for important intellectual content: Parakininkas, Werlin, Southern, Johnston, Kerschner.

Statistical analysis: Kelly, Johnston, Kerschner.

Obtained funding: Kerschner.

Administrative, technical, or material support: Parakininkas, Southern, Johnston, Kerschner.

Study supervision: Parakininkas, Werlin, Johnston, Kerschner.

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Additional Contributions: Aniko Szabo, PhD, provided biostatistics consultation, and Tina L. Samuels, MS, provided assistance in data collection and analysis.

REFERENCES


