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.
Prevalence of Pediatric AERD

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 laryngotracheal 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...
PRO000004759 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) 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 of bilaterally 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. Additional clinical features for these patients were also investigated and obtained during retrospective review of medical records, as given in Table 2. Half of the patients in the bronchoscopy group were being treated for gastroesophageal reflux, with 5 (15%) receiving H2 blockers (2 of whom had pepsin-positive BAL fluid samples), 12 (35%) receiving proton pump inhibitors (10 of whom had pepsin-positive BAL fluid samples), and 9 (9%) receiving metoclopramide (all of whom had pepsin-positive BAL fluid samples). More than half of the patients in the tracheostomy group were receiving antireflux medications, with 5 (16%) receiving H2 blockers (3 of whom had pepsin-positive BAL fluid samples) and 14 (45%) receiving proton pump inhibitors.

### 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. 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.

### 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 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.

Additional clinical features for these patients were also investigated and obtained during retrospective review of medical records, as given in Table 2.
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 fluids 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 presence for any LLMs or pepsin revealed a moderate agreement between the presence of LLMs and pepsin for the bronchoscopy and tracheostomy groups (κ = 0.3; P = .03) but no apparent agreement among controls (κ = −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 non-specific 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>
<thead>
<tr>
<th>Characteristic</th>
<th>Bronchoscopy Group (n = 34)</th>
<th>Tracheostomy Group (n = 31)</th>
</tr>
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<tbody>
<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>
<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>
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</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. Fischella 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.5,6,18

Farrell et al6 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.21 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.21 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%).23 In a large controlled study, Spechler et al24 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.25

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

Study supervision: Johnston, Kerschner.

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Additional Contributions: Previous Presentation:

Resources, National Institutes of Health.

The statistical support and interpretation of the collection, management, and analysis of the data.

consultant for Koufman Diagnostics LLC. No other

Study supervision: Parakininkas, Merle, Southern, Johnston, Kerschner.

Johnston, Kerschner.

Johnston, Kerschner.

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

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

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

Statistical analysis: Johnston, Kerschner.

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Study supervision: Parakininkas, Merle, Johnston, Kerschner.

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REFERENCES


