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The presence of pepsin in the lung and its relationship to pathologic gastroesophageal reflux

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Abstract

Background—Pepsin has been proposed as a biomarker of reflux related lung disease. It is the goal of this study to determine 1) if there is a higher reflux burden as measured by pH-MII in patients that are pepsin positive in the lung and 2) the sensitivity of pepsin in predicting pathologic reflux by pH, MII and EGD.

Methods—We recruited children between the ages of 1–21 with chronic cough or asthma undergoing bronchoscopy, esophagogastroduodenoscopy (EGD), and multichannel intraluminal impedance (pH-MII) probe placement. The reflux profiles were compared between those patients that were pepsin positive and negative; proportions were compared using Chi square analyses and means were compared using t testing.

Key Results—Only the mean number of non-acid reflux events was associated with pepsin positivity (0.04). The sensitivity and specificity of pepsin in predicting pathologic reflux by pH-MII or EGD was 57% and 65% respectively. The positive predictive value of pepsin in predicting pathologic reflux by pH, MII or EGD was 50% (11/22) and the negative predictive value was 71% (20/28). There was a significantly higher mean LLMI in patients that were pepsin positive compared to pepsin negative patients (81±54 vs. 47±26, p=0.001).

Conclusions and Inferences—Lung pepsin cannot predict pathologic reflux in the esophagus but its correlation with lung inflammation suggests that pepsin may be a important biomarker for reflux-related lung disease.

Keywords

Impedance; pepsin; nonacid reflux

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Introduction

Gastroesophageal reflux can exacerbate respiratory disease but the presence of reflux alone does not prove causality. To establish causality, most pediatric studies have focused on the correlation between respiratory symptoms and the presence of acidic gastroesophageal reflux, as measured by pH probe or on the presence of abnormal amounts of acid reflux in a 24 hour period. However, using pH measurements as a way to establish the association has proven problematic since 1) not all reflux is acidic and 2) distal reflux may not be the etiology of respiratory disease.

Recently, with the advent of multichannel intraluminal impedance with pH (pH-MII) which accurately measures acid and non-acid reflux and can pinpoint the height of the refluxate at 6 levels of the esophagus, pediatric studies have established that non-acid reflux is common in children and that full column reflux may be important in the genesis of respiratory symptoms. However, because there are no normal pH-MII values in children, it is not clear what amount and what type of reflux is associated with respiratory problems, and is most damaging. Furthermore, there are patients with respiratory disease that have a normal reflux burden who respond to anti-reflux surgery ¹. This suggests that even with pH-MII, catheter based reflux monitoring is imperfect to establish causality between gastroesophageal reflux and lung disease, and other tools to measure reflux related lung disease are needed.

One of these proposed tools is the measurement of pepsin in the lung. In limited previous studies, pepsin, which is produced in the stomach, has been found in the lungs of children and adults with respiratory disease suggesting the presence of microaspiration of gastric contents, but there has been an inconsistent relationship with lung pepsin and reflux monitoring ²⁻⁷. In a single adult study in which pH measurements were used, the sensitivity of salivary pepsin in predicting proximal esophageal reflux was 75% and the specificity was 91% ⁷. In a single pediatric study of children with reflux symptoms and respiratory disease, the authors found 84% of patients with respiratory disease and reflux symptoms were pepsin positive. In contrast, 87% of children with respiratory disease but had no reflux symptoms were pepsin negative ⁶. This data suggests that reliance on symptoms for the determination of reflux disease, or the use of pH monitoring to detect acid reflux alone is imperfect. Because of these limitations in the existing studies, the goal of this study was to determine in children 1) if there is a higher reflux burden as measured by pH-MII in patients that are pepsin positive in the lung compared to patients that are pepsin negative and 2) the sensitivity of pepsin in predicting reflux by pH, MII and EGD.

Material and Methods

This is a prospective, cross-sectional study of children between the ages of 1–21 who were presenting with a chief complaint of chronic cough or asthma to a tertiary care center. Patients were included if patients had a chronic cough (3 or more cough episodes per week for 3 or more months) or asthma (three or more asthma flares per year). Patients were recruited if they were undergoing bronchoscopy (at the recommendation of their pulmonologist) and esophagogastroduodenoscopy with biopsies and multichannel intraluminal impedance (pH-MII) probe placement in the operating room for the evaluation of their respiratory symptoms. Patients taking acid suppression therapy discontinued medications a minimum of 48 hours prior to the procedures. Patients were excluded from participation if they had prior esophageal or gastric surgery.

To determine a sample size calculation, we anticipated that the sensitivity of pepsin to detect pathologic reflux in the esophagus using pH-MII would be 90%, as the gold standard compared to 70% value when pH probe analysis alone is used since pH-MII detects between

30–89% more reflux episodes in children than pH probe alone. Based on these values, we estimated a sample size of 42 would provide 90% power, using an alpha of 0=0.05, to detect a 20% difference between the sensitivity of pepsin reported in the literature (70%) and the predicted sensitivity using pH-MII (90%). To allow for technical difficulties with sample processing or pH-MII malfunction, we recruited 50 patients.

Study protocol

Flexible bronchoscopy was performed under general anesthesia. The bronchoscope was wedged in a subsegmental bronchus (the location of which was chosen based on radiographic findings or gross findings during the exam) and 1 or 2 aliquots of 1 ml/kg of sterile normal saline was instilled and suctioned for pathologic analysis. The LLMI was determined by the same pathologist for each patient based on the methods outlined by Colombo and Hallberg⁸. According to this method, the lipid content of 100 consecutive macrophages is scored on a scale of 0–4 (where 0= no opacification and 4=completely opacified) and the scores are summed. 0.5 cc of bronchoscopy fluid were added to 0.25 cc of 100mM citric acid at pH 2.5 and samples were stored at –80 for later measurement.

After the bronchoscopy was performed, an attending gastroenterologist performed an esophagogastroduodenoscopy (EGD) with biopsies to assess for esophagitis. A minimum of 2 esophageal biopsies were obtained in the esophagus. Following the endoscopy, a pH-MII catheter was placed in the nose and the location was confirmed either with fluoroscopy or intraoperative chest xray. The catheters were adjusted following the ESPGHAN⁹ guidelines so that the pH sensor was at the third vertebral body above the diaphragmatic angle.

Each impedance study was performed using a portable pH-MII system (Sleuth, Sandhill Scientific, Denver, CO). All patients were admitted to Children's Hospital Boston for the 24-hour pH/MII recording. Three different, age appropriate impedance catheters were used in the study: infant (ages 0–2 years), pediatric (2–10 years) and adult (>10 years old). Impedance sensors were spaced 1.5 cm apart on the infant catheter, 2 cm apart on the pediatric catheter and 2–4 cm apart on the adult catheter.

Patients ate a regular diet with a minimum of three hours between each meal while in the hospital. Patients did not drink apple juice or soda during the study. When patients experienced symptoms or ate meals, they recorded their events on a symptom log and pushed the corresponding buttons on the recording device. Logs were reviewed by one of the authors (RR) during and at the end of the study to ensure accurate completion. All information from the logs was manually entered into the impedance tracing. Data collected during meals were excluded from the analysis.

Patients also completed a baseline history and symptom questionnaire which assessed the incidence of extraesophageal diagnoses in the 6 months prior to presentation at the specialist and the frequency of antibiotic and steroid use within the preceding 6 months.

The protocol was approved by the IRB and informed consent was obtained from each family and patient.

Reflux definitions

A pH probe was considered abnormal if the pH was less than 4 for greater than 6% of the time for children¹⁰. A normal pH-MII study was defined as < 73 reflux episodes per 24 hour period¹¹. A reflux episode detected by impedance was defined as a retrograde drop in impedance to more than 50% of baseline in the distal two channels. A series of these waves defines a reflux episode. As liquid passes over the catheter from the stomach into the esophagus, the impedance drops at the distal sensor (closest to the stomach). This wave

pattern progresses in a retrograde fashion up the catheter, resulting in subsequent visible drops in impedance. Bolus clearance time was defined as the time from a drop in impedance to 50% of its baseline value to its recovery to 50% of its baseline value in the distal most impedance channel. Acid reflux episodes are those episodes detected by both pH and impedance sensors. Non-acid episodes are those episodes detected by impedance sensors only. pH-only episodes are those episodes detected by the pH sensor only and are a minimum of 5 seconds in length. Full column reflux was defined as an episode that reached the highest pair of impedance sensors. The percentage of full column reflux was determined by dividing the number of full column events by the total number of reflux events. For each individual patient, the percentage of time that reflux is in the esophagus, as detected by impedance, was calculated by dividing the sum of the bolus clearance times in either the proximal or distal esophagus by the total study duration.

In this study, proportions were compared using Chi square analyses and means were compared using t testing or non-parametric equivalents.

Pepsin measurements

Total protein from clinical specimens were extracted in urea lysis buffer and protein content was measured by Bradford assay (500-0006, BioRad, Hercules, CA)¹². 30 µg total protein, or less in the case of low concentration specimens, was loaded on 10% SDS-PAGE gels according to standard SDS-PAGE protocol. Purified human pepsin 3b (isolated from human gastric juice by ion exchange chromatography) and human pepsinogen I (P1490, Sigma, St. Louis, MO) were run alongside clinical specimens as positive and negative controls, respectively¹³. Proteins were transferred to PVDF membrane (RPN2020F, GE Healthcare, Piscataway, NJ) and probed with rabbit anti-pepsin antibody diluted 1:350 and mouse anti-β-actin antibody (CP01, EMD Chemicals, Gibbstown, NJ) diluted 1:5,000¹⁴. Blots were then probed with appropriate peroxidase-conjugated secondary antibody diluted 1:5,000 (P0447/P0448, Dako, Copenhagen, Denmark). All antibodies were diluted in phosphate buffered saline, 0.1% Tween-20, and 5% nonfat dry milk. Blots were exposed to enhanced chemiluminescence reagents (sc-2048, Santa Cruz Biotechnology, Santa Cruz, CA) followed by radiographic exposure and development.

To insure that pepsin was not synthesized locally in the lung, fifteen microliters BAL fluid from four randomly selected patients (BAL1-4) was separated via SDS-PAGE and transferred to polyvinylidene fluoride membrane. Presence of pepsin in BAL fluid was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/western blot (Figure 1 A). Human pepsin 3b and pepsinogen I were included as positive and negative controls. Pepsin was detected using affinity purified antibody described previously. Pepsin antibody did not react with human pepsinogen I. Pepsin was detected in all samples. To verify that pepsin observed by SDS-PAGE/western blot was not synthesized locally, RNA was extracted from remaining BAL fluid and pepsinogen mRNA was detected by reverse transcriptase polymerase chain reaction (RT-PCR) (Figure 1 B). Human gastric or BAL cDNA reverse transcribed from 250ng DNase-treated RNA was PCR amplified using primers for human pepsinogen A (forward: ACCGTGGACAGCATCACCATG, reverse: TCTTCCTGGGAGGTGGCTG, 30 cycles, 62°C annealing). The housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1), was amplified as a positive control (forward: TGCTCGAGATGTGATGAAGG, reverse: CCTGACCAAGGAAAGCAAAG, 35 cycles, 55°C annealing). Primers were designed to span >100bp introns. Amplicon was separated on 2% agarose with 50–1000bp DNA Marker (Cambrex, East Rutherford, NJ). Amplicon corresponding to pepsinogen A (437bp, expected size) was detected in human gastric tissue, but not in BAL specimens. HPRT1 was detected in all samples (307bp, expected size) (Figure 1).

Results

Fifty patients were recruited for participation in the study. The mean age was 67 ± 43 months. The reflux profiles for the patients are shown in table 1. We then divided patients into pepsin positive and negative groups; twenty two patients (44%) were pepsin positive. The differences in demographics between pepsin positive and negative patients are shown in table 2; there was no difference in the frequency of extraesophageal symptoms in patients who were pepsin positive or negative. Table 3 shows the comparison of reflux parameters between pepsin positive and negative patients. As can be seen, patients who were pepsin positive had more non-acid reflux than patients who were pepsin negative. There was no difference in any other reflux parameter including full column reflux. There was also a positive correlation between pepsin concentration and the number of non-acid reflux events ($r=0.318$, $p=0.02$) but there was no significant correlation between other reflux parameters and pepsin ($p>0.2$).

Table 4 shows the relationship between standard diagnostic definitions of pathologic reflux by different methodologies and pepsin positivity; there is no significant relationship between pepsin positivity and an abnormal endoscopy, pH probe or impedance. There was a significantly higher mean LLMI in patients that were pepsin positive compared to pepsin negative patients (81 ± 54 vs. 47 ± 26 , $p=0.001$). There was no difference in the lung neutrophil burden between pepsin positive and negative respectively (13 ± 11 vs. 11 ± 9 , $p=0.6$).

Three patients had a history of aspiration on swallow studies after their procedures. There was no difference in the rate of pepsin positivity, the amount of reflux, the LLMI or the neutrophil counts in patients with and without a history of aspiration during swallowing.

The sensitivity and specificity of pepsin in predicting reflux disease are shown in Table 5. The positive predictive value of pepsin in predicting pathologic reflux by pH, MII or EGD was 50% (11/22) and the negative predictive value is 71% (20/28). When we limited our sample size to only those patients with gastrointestinal symptoms, namely abdominal pain or a sensation of food coming up into their mouth, the sensitivity of pepsin was 70% (using a positive pH, MII or EGD as the gold standard), the specificity was 81%, and the positive predictive value was 54%.

We measured pepsin mRNA in bronchoscopy fluid in 4 randomly selected pepsin positive samples and found none contained pepsinogen A mRNA present (Figure 1). Pepsinogen A was detected in human gastric tissue, but not in BAL specimens.

Discussion

Pepsin in the lung has been proposed as a biomarker for reflux-related lung disease. We found that 44% of our patients had pepsin in the bronchoscopy fluid, and pepsin presence did not correlate with any reflux parameter except non-acid reflux burden. When we determined the sensitivity of pepsin relative to other reflux testing, the sensitivity and the specificity were universally low suggesting that pepsin may not be an adequate marker for reflux related lung disease or that the gold standard tools for evaluating reflux (pH probe, pH-MII and endoscopy) are not ideal gold standards upon which sensitivity analyses are based.

Previous pediatric studies have explored the importance of pepsin in predicting reflux related lung disease. Krishnan et al. studied 64 children with reflux symptoms who did and did not have respiratory symptoms⁶. The authors found that the sensitivity of tracheal pepsin, using pH probe as the gold standard, was 78% in children with reflux and respiratory

symptoms. Using esophagitis as the gold standard, they found that the sensitivity of pepsin was 83%⁶. In another study of sputum samples in adults with GER symptoms undergoing pH monitoring, Potluri et al. report that the sensitivity of pepsin in sputum at any given time, using a pH drop to <4 as the gold standard reflux test, was 75% and specificity 91% for proximal acid reflux while it had a sensitivity of 63% and a specificity of 92% for distal acid reflux⁷.

The sensitivity and specificity numbers we report here are lower than those found by those previous authors. We postulate that, as compared with Krishnan et al, we chose all patients who presented for bronchoscopy for the evaluation of reflux disease and did not limit our sample to patients only with reflux symptoms. We also think our results differ from those reported by Potluri et al. because the patients they studied also had known GER symptoms and patients were producing a sputum sample when they were experiencing a symptom. When we limited our sample size on only patients with abdominal symptoms (abdominal pain or regurgitation) to see if the results were similar to the above mentioned studies, the sensitivity (70%) and specificity of pepsin (81%) which were comparable. Therefore our data reinforces the importance of the clinical history; patients with reflux symptoms are more likely to have extraesophageal symptoms related to reflux.

Therefore, we feel that our data represents the sensitivity of the test in unselected patients presenting with extraesophageal symptoms for an aerodigestive evaluation. Overall, however, the positive predictive value of pepsin in our population was only 50% suggesting that it cannot be used as a sole test for reflux disease. While the sensitivity can be improved in those patients with gastrointestinal symptoms, arguably the pepsin is not necessary clinically if it is clear the patient is symptomatic from a gastrointestinal perspective.

The only component that correlated with pepsin positivity was non-acid reflux burden. While pepsin is inactivated between pH 4–5, its presence represents a biomarker for reflux-respiratory disease independently of its activity, given the fact that it is secreted in the stomach. We have previously shown that non-acid reflux is more highly associated with respiratory symptoms which may be because non-acid reflux is not sensed by the patient until it reaches the upper esophagus which, by that time, has exposed the airway to refluxate and therefore to pepsin¹⁵. We hypothesize that this association with non-acid reflux and pepsin positivity occurs because non-acid reflux may not be sensed until it reaches the proximal esophagus or oropharynx at which point the refluxate is already exposed to the airway. Interestingly, in the present study, we did not find an association between full column reflux and pepsin positivity which again may suggest that our tool for measuring full column reflux (pH-MII) is flawed or that it may only take a small number of full column events for the airway to become pepsin positive; in this study, alone, 46% of all reflux events are full column and prior studies have shown that 30–40% of reflux is full column^{11,15}.

In this study, there was a significantly higher LLMI in patients that are pepsin positive than patients who are pepsin negative. LLMI has been previously shown not to correlate with reflux by pH-MII but it may be a marker of lung disease severity and has been elevated in patients with asthma, aspiration, lung tumors and other chronic respiratory diseases^{16–20}. Also, recent studies have suggested that lung injury may result in increased production of pepsinogen C which is produced in the lung and prior pepsin studies have been potentially confounded by the cross reactivity of the pepsin antibodies in the assay with pepsinogen C^{21,22}; we hypothesized that if the LLMI was elevated signifying lung injury, we might falsely see pepsin positivity due to the presence of pepsinogen C. To address this issue, we analyzed 4 pepsin positive bronchoscopy samples for the presence of pepsinogen A and there was none present in any of the samples suggesting that our assay was detecting pepsin

synthesized from the gastrointestinal tract rather than locally synthesized. One possibility to explain the higher LLMI in pepsin positive patients is that pH-MII and endoscopy reflect total reflux burden and that microaspiration may be occurring, even with a normal reflux burden. One would expect, then, that pepsin positive patients would have a more favorable outcome with anti-reflux procedures and future studies are needed to determine how pepsin positive patients with a normal reflux burden respond to reflux therapies. This also suggest that new biomarkers are needed and that the gold standard tools that we use to diagnose reflux should be used in context with the clinical picture and lung markers of reflux disease.

There are several limitations of this study. First, we used pH-MII testing and EGD as gold standard tests to evaluate for reflux related lung disease but the true gold standard method of proving reflux related lung disease is the performance of fundoplication in patients with resultant symptomatic improvement. In children, this is particularly difficult to do so we are using the standard tools to assess reflux burden but these may not be the best tools to establish causality from reflux. Nevertheless, since these are the tools use by gastroenterologists, we feel that they accurately reflect the current clinical practice.

A second limitation is the possibility that our assay is actually detecting lung pepsinogen c rather than gastric pepsin alone. To address this, we performed mRNA analysis of the bronchoscopy fluid to assess for the presence of pepsinogen A which was not in fact present suggesting that the pepsin that was present was of gastric origin so we feel this potential confounder is unlikely. Additionally, the antibody used in our assay does not detect gastricsin protein which is synthesized from pepsinogen C. Therefore, our detection of pepsin is not a result of cross reactivity with pepsinogen C¹⁴. The porcine antibodies used in other studies do likely cross-reacted with gastricsin and thus pepsin positivity may be associated with lung inflammation and the production of pepsinogen C.

In conclusion, pepsin positivity is high in patients with respiratory disease and the sensitivity of pepsin using gold standard reflux tests is low. Pepsin does seem to correlate with the LLMI which raised the possibility that aspiration may be occurring even with normal reflux burdens and future outcome studies are needed to determine if pepsin predicts response to reflux treatments.

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Abbreviations used in this paper

pH-MII	multichannel intraluminal impedance
GERD	gastroesophageal reflux disease

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Figure 1. Western blot showing the presence of pepsin and the lack of pepsinogen A in BAL fluid

A.- Pepsin was detected in all 4 BAL samples. Human pepsin 3b and pepsinogen I were included as positive and negative controls. The pepsin antibody did not react with human pepsinogen I.

B.- Pepsinogen A was detected in human gastric tissue, but not in BAL specimens. HPRT1 (house-keeping gene) was detected in all samples. This confirms that the pepsin in the BAL fluid was not synthesized locally.

(BAL= bronchoalveolar lavage; HPRT1 : hypoxanthine-guanine phosphoribosyltransferase 1)

Table 1

Reflux profiles of 50 patients.

Acid Events	26 ± 15
Non-Acid Events	18 ± 15
pH only Events	7 ± 6
% Full column events	46 ± 20
% pH<4	3.9 ± 4.6
% Proximal Reflux	0.6 ± 0.7
% Distal Reflux	1.6 ± 1.7

Table 2

Relationship between symptoms and diagnoses in the preceding 6 months and pepsin positivity.

	Pepsin Negative n=28	Pepsin Positive n=22	P Value
Abdominal Pain	12/26	11/19	0.7
Chest Pain	4/28	3/20	0.7
Ear Infections	11/28	3/19	0.4
Ear Tubes	4/28	5/22	0.4
Tonsillectomy	6/28	7/22	0.3
Sinus Infection	6/26	6/17	0.3
Pneumonia	6/26	8/19	0.4
Croup	5/27	4/20	0.9
Asthma	25/28	12/19	0.03
Cough	24/28	13/22	0.48
ICU admission	6/28	2/20	0.2

Table 3

Reflux profiles of pepsin positive and pepsin negative patients.

	Pepsin Negative n=28	Pepsin Positive n=22	P Value
Acid Events	28 ± 14	23 ± 15	0.2
Non-Acid Events	14 ± 10	23 ± 19	0.04
Total Events	41 ± 20	46 ± 29	0.6
pH only Events	8 ± 5	7 ± 7	0.5
% Full Column Events	46 ± 21	47 ± 20	0.7
% Time pH<4	3.7 ± 3.7	4.2 ± 5.6	0.9
% Proximal Reflux	0.6 ± 0.7	0.6 ± 0.5	0.9
% Distal Reflux	1.6 ± 1.9	1.6 ± 1.3	0.9

Table 4

Relationship between pepsin positivity and the number of patients with abnormal reflux testing.

	Pepsin Negative	Pepsin Positive	P value
Abnormal pH probe	6/28	5/22	0.9
Abnormal MII	2/28	5/22	0.1
Esophagitis	6/28	3/22	0.7

Table 5

Sensitivity of pepsin using different reflux testing as a gold standard.

	Sensitivity of Pepsin	Specificity of Pepsin
If Abnormal EGD	67%	59%
If Abnormal pH probe	45%	56%
If Abnormal MII	71%	60%
If Any Abnormal Test (pH/MI/EGD)	57%	65%