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Pepsin detection in patients with laryngopharyngeal reflux before and after fundoplication

Eelco Wassenaar · Nikki Johnston · Albert Merati · Martin Montenovo · Rebecca Petersen · Roger Tatum · Carlos Pellegrini · Brant Oelschlager

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Abstract
Background Some patients with gastroesophageal reflux disease (GERD) suffer from laryngopharyngeal reflux (LPR). There is no reliable diagnostic test for LPR as there is for GERD. We hypothesized that detection of pepsin (a molecule only made in the stomach) in laryngeal epithelium or sputum should provide evidence for reflux of gastric contents to the larynx, and be diagnostic of LPR. We tested this hypothesis in a prospective study in patients with LPR symptoms undergoing antireflux surgery (ARS).
Methods Nine patients undergoing ARS for LPR symptoms were studied pre- and postoperatively using a clinical symptom questionnaire, laryngoscopy, 24-h pH monitoring, biopsy of posterior laryngeal mucosa, and sputum collection for pepsin Western blot assay.
Results The primary presenting LPR symptom was hoarseness in six, cough in two, and globus sensation in one patient. Pepsin was detected in the laryngeal mucosa in eight of nine patients preoperatively. There was correlation between biopsy and sputum (+/+ or −/−) in four of five patients, both analyzed preoperatively. Postoperatively, pH monitoring improved in all but one patient and normalized in five of eight patients. Eight of nine patients reported improvement in their primary LPR symptom (six good, two mild). Only one patient (who had negative preoperative pepsin) reported no response to treatment of the primary LPR symptom. Postoperatively, pepsin was detected in only one patient.
Conclusions Pepsin is often found on laryngeal epithelial biopsy and in sputum of patients with pH-test-proven GERD and symptoms of LPR. ARS improves symptoms and clears pepsin from the upper airway. Detection of pepsin improves diagnostic accuracy in patients with LPR.

Keywords Pepsin · Laryngopharyngeal reflux · LPR · Antireflux surgery · Fundoplication · Gastroesophageal reflux · GERD · Laryngoscopy

E. Wassenaar (✉) · M. Montenovo · R. Petersen · R. Tatum · C. Pellegrini · B. Oelschlager
Department of Surgery, University of Washington, 1959 NE Pacific Street, Box 356410, 98195 Seattle, WA, USA
e-mail: ewassena@uw.edu

N. Johnston
Department of Otolaryngology, Medical College of Wisconsin, Milwaukee, WI, USA

A. Merati
Department of Otolaryngology-Head and Neck Surgery, University of Washington, Seattle, WA, USA

While most patients with gastroesophageal reflux disease (GERD) suffer from typical esophageal manifestations such as heartburn and regurgitation, there is a subset of patients who suffer from so-called laryngeal symptoms, which may be caused by laryngopharyngeal reflux (LPR) [1, 2]. The clinical spectrum of LPR is vast and includes symptoms due to laryngeal irritation and inflammation such as cough, hoarseness, subglottic stenosis, globus sensation, and laryngeal cancer [3]. To date, diagnosis of this entity has focused on identification of injury (by laryngoscopy), traditional measures of gastroesophageal reflux (esophagoscopy or pH monitoring), pharyngeal pH monitoring or empiric treatment of symptoms alone by control of gastric acid secretion (proton pump inhibitors). These methods are not specific, and thus are not reliable to diagnose LPR nor can they predict response to antireflux

therapy [4]. Most laryngeal diseases associated with LPR are thought to develop following direct contact of the laryngeal epithelium with gastric refluxate containing not only acid but also pepsin, bile acids, and other components [5]. This may explain why patients with suspected LPR who are prescribed proton pump inhibitors (PPIs) are less likely to respond to antireflux therapy than those with typical symptoms of GERD [6]. In fact, acid may be less important in this entity than it is for typical GERD symptoms [7]. Pepsin, for example, may contribute to LPR injury. In addition, because, like acid, pepsin is only made in the stomach, it may be a good marker for laryngeal reflux, and the finding of this molecule in the larynx may be a proxy for aspiration of gastric contents [8]. Recently, it has been reported that pepsin is taken up by human laryngeal epithelial cells by means of receptor-mediated endocytosis in patients with a clinical diagnosis of LPR [9].

Antireflux surgery (ARS), by creating a mechanical barrier to reflux, in theory should be more effective than acid suppression in treating LPR as it reduces not only acid but also all other components of gastroesophageal reflux. However, surgical therapy, so successful in the resolution of heartburn and regurgitation, has been less successful in treating disorders thought to be associated with LPR [10, 11]. The inferior efficacy of ARS in patients with symptoms of LPR as compared with those who suffer from typical GERD symptoms may, in part, be due to the lack of accuracy in the diagnosis of LPR.

We tested the hypothesis that pepsin detection in patients with symptoms suspected to be due to LPR may increase the accuracy of diagnosis. We tested this hypothesis by measuring pepsin in laryngeal biopsies and in sputum in a group of patients undergoing ARS with the intent of determining: (1) the incidence of pepsin detection in patients with LPR, (2) the concordance between pepsin detection in laryngeal biopsy specimens and sputum, (3) the ability of ARS to eradicate pepsin from the upper airway, and (4) the relationship of the presence of pepsin, pre- or postoperatively, to the presence of symptoms of LPR.

Patients and methods

Patients

We studied 10 consecutive patients referred to the Department of Surgery from the Department of Otolaryngology of the University of Washington between October 2008 and November 2009, who were presumed to have LPR-induced larynx injury or disease (using traditional diagnostic criteria) and in whom a decision had been made to perform ARS.

Inclusion criteria

1. Documented GERD by abnormal 24-h pH monitoring, defined as DeMeester score > 14.7
2. Presence of severe LPR symptoms, including hoarseness, coughing, sore throat, and/or globus sensation
3. Symptom severity scale for the patient's primary symptom ≥ 6 out of 10 on the preoperative questionnaire
4. Decision to perform ARS for LPR prior to enrollment in the study

Exclusion criteria

1. Previous operation of the esophagus or stomach
2. Inability to complete the symptom questionnaire

Patients were seen and evaluated according to a standardized protocol before and 6 months following ARS, with the exception that postoperatively no laryngeal biopsies were taken.

Perioperative evaluation

Symptom questionnaire

All patients filled out a preoperative symptom questionnaire that assessed frequency and severity of symptoms. The questionnaire focused on the presence of laryngeal symptoms thought to be related to LPR as well as more typical GERD symptoms. All symptoms were scored based on frequency, with 0 indicating "never" and 4 indicating "multiple times a day"; severity was evaluated using a 10-point symptom severity score (SSS), with 0 indicating "no symptoms" and 10 indicating "worst." The primary LPR symptom was defined as the symptom that was present at the highest frequency and highest severity, with the frequency being the more important indicator. Improvement in the primary LPR symptom postoperatively was defined as good if an improvement of at least 2 points was recorded on the frequency scale [e.g., a decrease from multiple times a day (score 4) to once per week or less (score 2)], as mild if lesser improvement was noted in either frequency or severity, and as none if no improvement or worsening was recorded.

pH monitoring

Twenty-four-hour pH monitoring testing was performed on all patients off PPIs. Abnormal esophageal acid exposure was defined as DeMeester score > 14.7.

Laryngoscopy

Flexible laryngoscopy was performed to examine the pharynx and larynx. The Reflux Finding Score (RFS) was determined using the criteria of Belafsky [12]. RFS > 7 was considered positive or likely to be related to reflux. After visual examination, 2–3 grasp biopsies were taken with a 1-mm cup biopsy forceps from the squamous epithelium of the interarytenoid area of the posterior larynx. All biopsies were taken under general anesthesia immediately before the start of ARS. Biopsies were placed in tubes on dry ice and sent to the Medical College of Wisconsin laboratory for pepsin immunoassay testing.

Sputum collection

Subjects were given 30-ml tubes for the collection of sputum/saliva. Subjects were instructed to cough a few times to clear the sputum or saliva from the back of their throat and then spit it into the tubes. The tubes were closed and snap-frozen upon collection using liquid nitrogen. The tubes were removed from liquid nitrogen after 5 min and stored at −80°C until shipping. Tubes were shipped via overnight priority courier on dry ice and cold packs to the Medical College of Wisconsin laboratory for pepsin immunoassay testing.

Western blot analysis for pepsin

Total protein from sputum and laryngeal biopsy specimens was extracted in urea lysis buffer and protein content measured by Bradford assay. Thirty micrograms total protein was loaded onto 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels according to standard protocol. Purified human pepsin 3b (isolated from human gastric juice by ion-exchange chromatography [13]; Medical College of Wisconsin Institutional Review Board Study Protocol Number: PRO00004759) and human pepsinogen I (Sigma, St. Louis, MO) were run alongside clinical samples as positive and negative controls, respectively. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ) and probed with rabbit anti-pepsin antibody (1:350) and mouse anti-β-actin antibody (1:5,000; EMD Chemicals, Gibbstown, NJ). Blots were then probed with appropriate peroxidase-conjugated secondary antibody diluted 1:5,000 (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 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by radiographic exposure and development. The specificity and sensitivity of the polyclonal anti-human pepsin have been described [14, 15]. The density of the pepsin band was recorded in semiquantitative fashion as absent (0, no pepsin detected), low (+, less than half the density of the positive control), moderate (++, the same density as the positive control) or high (+++, more than twice the density of the positive control). All pepsin testing was performed at the Medical College of Wisconsin and is not commercially available.

Operation

Patients underwent ARS by either laparoscopic Nissen fundoplication or endoluminal fundoplication (EsophyX®; EndoGastric Solutions®, Redmond WA) when patients: (1) had hiatal hernia ≤ 2 cm, and (2) chose this operation over the Nissen fundoplication. Both procedures were done under general anesthesia. Laparoscopic Nissen fundoplication was performed by one of two experienced esophageal surgeons (B.O. and C.P.) as previously described by our group [16]. Endoluminal fundoplication was performed by one surgeon (B.O.) as described by Bell and Cadière [17].

Statistical analysis

Data were collected prospectively and analyzed using STATA version 11.0 (Stata Corp., College Station, TX). Continuous and ordinal values were summarized as medians with ranges, and categorical variables were summarized as counts and frequencies. A Wilcoxon matched-pairs signed-rank test and/or Spearman's rank correlation coefficient were used to compare preoperative and postoperative ordinal variables. p Value < 0.05 was considered significant.

This study was approved by the University of Washington Human Subjects Division under HSD # 34459.

Results

Patient characteristics

Seven women and three men were included in this study. One patient withdrew from the study before any postoperative testing had been done, therefore data for nine patients were available for analysis. Median age was 54 years (range 42–71 years) with median BMI of 31 kg/m^2 (23–39 kg/m^2). Seven patients underwent laparoscopic Nissen fundoplication, and two patients (no. 7 and 8) had endoluminal fundoplication (Esophyx®). Median follow-up was 8 months (5–16 months). Because symptoms remained and DeMeester score did not normalize, one of these patients (no. 8) with Esophyx® underwent subsequent laparoscopic Nissen fundoplication. Results presented are after initial procedure, unless otherwise stated.

Pepsin biopsy and sputum

Nine patients underwent biopsy of the posterior larynx (postcricoid mucosa), and preoperative sputum was analyzed in five. Pepsin was detected in eight of nine patients preoperatively. In four of the five patients who had both biopsies and sputum available for analysis preoperatively, there was a correspondence between pepsin detection in tissue and sputum (+/+ or −/−). All nine patients had postoperative sputum testing, and only one patient (no. 7) still had pepsin detected in her sputum, although the amount had decreased (from +++ to +). The patient in whom pepsin was not detected preoperatively also had a negative pepsin assay after surgery. Results of preoperative studies are presented in Table 1.

pH monitoring and pepsin

All patients had an abnormal DeMeester score on pH monitoring before surgery, with a median of 22.5 (17.9–58.2). Of the eight patients with available postoperative pH testing results, five had normalization of their pH score. All but one of the patients with normalization of reflux as measured by pH monitoring had no detection of pepsin postoperatively, and in this patient there was a decrease in the amount of pepsin detected compared with preoperative assay. All three patients who had abnormal pH studies after surgery had absence of pepsin in their sputum postoperatively (Table 1).

Laryngoscopy and pepsin

Preoperative RFS was available in seven patients with a median score of 9 (5–21). Postoperative RFS decreased in all six patients who underwent repeat laryngoscopy, with median score of 6 (4–18) ($p < 0.03$). There was little relationship between preoperative RFS and detection of pepsin. In fact, the patient with no pepsin detected preoperatively had the highest RFS score (Table 1).

Table 1 Pre- and postoperative results of pH testing and pepsin detection in biopsy and sputum

Patient	Pre RFS	Post RFS	Pre DMS	Post DMS	Pre biopsy	Pre sputum	Post sputum	Improvement
1	9	5	23.2	0.3	+++	NA	−	Good
2	NA	NA	22.5	42.0	+++	NA	−	Good
3	NA	NA	22.1	10.2	+	NA	−	Good
4	11	6	44.2	6.4	+++	NA	−	Good
5	16	8	58.2	4.4	+	−	−	Mild
6	5	4	17.9	15.2	+	+++	−	Good
7	9	6	19.8	7.0	++	+++	+	Good
8	21	18	20	19.4	−	−	−	No
9	6	NA	24	NA	+	+++	−	Mild
Median	9	6	22.9	8.6				

Pre RFS preoperative reflux finding score, *Pre DMS* preoperative DeMeester score (normal < 14.7), *Post DMS* postoperative DeMeester score, *Pre biopsy* preoperative pepsin detection in biopsy, *Pre sputum* preoperative pepsin detection in sputum, *Post sputum* postoperative pepsin detection in sputum, *Improvement* primary symptom improvement, *NA* not available

Table 2 Pre- and postoperative frequency and severity of primary LPR symptom

Patient	Primary symptom	Pre freq	Post freq	Pre sev	Post sev	Improvement
1	Hoarseness	4	1	10	3	Good
2	Hoarseness	4	0	8	NA	Good
3	Hoarseness	4	2	10	3	Good
4	Hoarseness	4	2	7	3	Good
5	Hoarseness	4	4	10	6	Mild
6	Globus	2	0	6	0	Good
7	Cough	4	0	9	0	Good
8	Hoarseness	4	4	7	9	No
9	Cough	4	4	10	9	Mild

Pre freq preoperative frequency (0 = never, to 4 = several times a day), *Pre sev* pre-operative severity (0 = none, to 10 = worst); *NA* not available

Correlation of symptoms with testing

Pre- and postoperative primary symptom frequency and severity are presented in Table 2. The most common primary LPR symptom was hoarseness ($n = 6$); two patients reported cough and one globus sensation. Postoperatively, six patients had good symptom improvement, two had mild improvement, and one had no improvement. We evaluated the correlation between preoperative diagnostic studies (pH monitoring, laryngoscopy, and pepsin) for potential predictive trends.

All patients with normalization of their DeMeester score ($n = 5$) had symptom improvement; however, one of these patients had only mild improvement. Only one of the three patients without normalization of their DeMeester score following surgery had no symptom improvement, while the other two patients reported good symptom improvement despite ongoing abnormal acid exposure (one slightly improved and one worsened) in the distal esophagus.

The three patients with normalization of the RFS postoperatively had good symptom improvement. Of the two patients without normalization of RFS, one had mild improvement and one did not have any improvement. Of the patients with normal preoperative RFS, one had good improvement and one had mild improvement.

All eight patients with positive preoperative pepsin assay had symptom improvement (six good, two mild). Seven of these patients had no pepsin detected postoperatively, and one had a decrease in the amount of pepsin detected. The patient who did not have pepsin detected preoperatively, nor postoperatively, did not have symptom improvement, despite normalization of the DeMeester score after Nissen fundoplication.

Discussion

This study is the first to study pepsin detection in patients before and after ARS for LPR. Our findings strongly suggest that the measurement of pepsin in patients with GERD and laryngeal symptoms improves the accuracy of diagnosis of LPR. Indeed, we found that, in patients with documented GERD and signs and symptoms of LPR, pepsin is frequently detected in the airway (by biopsy or sputum), providing further evidence for proximal reflux of gastric contents as the primary mechanism of disease. Secondly, there was good concordance between pepsin detection in biopsy and sputum specimens, which is encouraging for practical application of the test, since sputum collection is much easier to perform than laryngeal biopsy. We also observed that ARS was successful in eradicating pepsin from the upper airway and that the presence of pepsin preoperatively and its elimination by ARS is associated with resolution of symptoms. As a result, pepsin seems to hold real promise in the diagnostic armamentarium for patients with LPR.

ARS provides relief of heartburn and regurgitation in over 90% of patients with GERD. By contrast, ARS is less effective in the treatment of laryngeal symptoms. There are at least two reasons for this observation: First, laryngeal injury probably is a reflection of multifactorial etiology, and secondly, there is not an accurate test to positively diagnose LPR [4]. Symptoms associated with LPR are not specific enough to determine which patients truly suffer from LPR. Heartburn, the most common symptom present in GERD patients, is often absent in these patients, and the laryngeal symptoms may be due to any number of factors other than gastroesophageal reflux [18]. The predictive value of laryngoscopy is variable, although its accuracy increases in centers with expertise and volume [19, 20]. Esophageal pH monitoring is generally considered the gold standard for GERD, but does not have strong predictive value for response to therapy in LPR patients [21]. The mechanism of disease in LPR is presumed to be direct contact of the larynx and pharynx with gastric contents in most cases. As a result, pH monitoring has been applied to the pharynx as a method for measuring aspiration events, but this is a difficult environment in which to measure pH accurately. Also, refluxate that makes it to this level may not have the same acid composition as is found more distally above the lower esophageal sphincter (LES). The fact that we detected pepsin in eight of the nine patients who we suspected of having LPR is, in itself, a promising finding. It not only lends further evidence for laryngeal and pharyngeal contact of reflux in LPR, but also suggests that pepsin assay may be sufficiently sensitive to use as a diagnostic test.

For pepsin detection to be clinically applicable as a diagnostic test, a specimen needs to be easy to collect. In four out of the five patients with both preoperative biopsy and sputum analysis there was concordance in pepsin detection (+/+ or −/−). Therefore, the sensitivity of laryngeal biopsy seems to be similar to that of sputum collection, a less invasive method that can be easily and rapidly performed in an outpatient setting. More evidence will need to be obtained, however, before we can conclude that sputum collection is equivalent to biopsy or how best to collect these specimens. Still, the practical application of pepsin detection will be highly dependent on its ease of measurement.

The reason that ARS is likely more effective than pharmacologic acid reduction in the treatment of LPR is its ability to reduce all constituents of gastric reflux. Not surprisingly, therefore, ARS seems to be very effective in eradicating pepsin from the upper airway. Even more important though is whether the presence of pepsin predicts

response to ARS. Our study strongly suggests that this may be the case. Of the nine patients available for follow-up, only one patient reported no improvement in the primary LPR symptom. This patient had an abnormal pH monitoring study (DeMeester score 20) and abnormal laryngoscopy (RFS = 21) preoperatively, but was the only patient who had negative pepsin in both the laryngeal biopsy and sputum specimens preoperatively. Even though we could document excellent control of gastroesophageal reflux with pH monitoring postoperatively, no improvement in hoarseness (the primary LPR symptom) was reported. Therefore, the absence of pepsin detection in this patient's specimens may be evidence that LPR was not the etiology of upper respiratory symptoms. Although this pilot study is not powered to draw definitive conclusions, neither pH monitoring nor laryngoscopy seemed to correspond with symptomatic response to ARS as well as pepsin.

All patients with preoperative pepsin detection in either sputum or biopsy had absence of pepsin in their sputum postoperatively, except for one patient who had a significant decrease. Since it is unknown how long it takes for pepsin to be eliminated from laryngeal cells, it may be possible that in this particular patient elimination of pepsin could be demonstrable after a longer follow-up period.

This study shows that pepsin detection in laryngeal cells has the potential to be an accurate diagnostic test for LPR, clearly paving the way for further investigation. As a result, we are using these data to organize a larger, multicenter trial to elucidate the best way for detecting pepsin, its reliability as a marker for LPR, and its predictive value for response to antireflux therapy.

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