Comparison of Impedance and Pepsin Detection in the Laryngeal Mucosa to Determine Impedance Values that Indicate Pathological Laryngopharyngeal Reflux

Martin Formánek, MD, PhD¹, Debora Jančátová, MD¹, Pavel Komínek, MD, PhD, MBA¹, Radoslava Tomanová, MD² and Karol Zeleník, MD, PhD¹

OBJECTIVE: Recently, a 24-h impedance was used to detect laryngopharyngeal reflux (LPR). However, not every case of LPR is pathological. Thus, pathological pharyngeal impedance values need to be clearly established to diagnose pathological LPR. The aim of our study was to establish pathological 24-h pharyngoesophageal impedance/pH values for the diagnosis of LPR.

METHODS: The study was conducted in a tertiary care setting. A total of 30 patients who were referred to microlaryngoscopy for a laryngeal pathology that might be caused by LPR were included in this prospective study. All patients were off proton-pump inhibitor therapy. The 24-h pharyngoesophageal impedance–pH monitoring was performed 1 day before surgery. A biopsy of laryngeal tissue was obtained during microlaryngoscopy and was analyzed by immunohistochemistry to detect pepsin. The patients were divided into two groups: pepsin negative and pepsin positive (which indicated pathological LPR). The results of 24-h multichannel intraluminal impedance–dual-channel pH monitoring were compared between the groups. The number of LPR episodes in the pepsin-positive group was analyzed to establish a cutoff value for pathological LPR.

RESULTS: There were 18 participants in the pepsin-negative group and 12 in the pepsin-positive group. The median total pharyngeal refluxes detected were two (0–5) in the pepsin-negative group and 14 (6–39) in the pepsin-positive group (P < 0.001), although the groups were otherwise homogeneous. There was a statistically significant difference in the number of all types of refluxes between groups. Six or more pharyngeal refluxes were the cutoff for the presence of pepsin in the laryngeal mucosa and, thereby, for the diagnosis of relevant/pathological LPR.

CONCLUSION: Six or more pharyngeal reflux episodes registered during the 24-h impedance/pH monitoring seem to be the cutoff for diagnosing pathological LPR. Therefore, it is possible to suggest establishing this value as the pathological impedance value indicating pathological LPR. These results must be interpreted with caution due to the small sample size.

Clinical and Translational Gastroenterology (2017) 8, e123; doi:10.1038/ctg.2017.49; published online 26 October 2017

Subject Category: Esophagus

INTRODUCTION

Laryngopharyngeal reflux (LPR) is defined as the reflux of (duodeno)gastric contents above the upper esophageal sphincter. The protecting mechanisms in nonesophageal mucosa are insufficient; therefore, LPR can cause or facilitate many pathologies of the aerodigestive tract, such as chronic laryngitis and pharyngitis, acute or chronic otitis media, and rhinosinusitis.¹–⁴ Many novel methods have recently been available for diagnosing LPR. However, an ideal method does not exist. Currently, 24-h multichannel intraluminal esophageal impedance or pH monitoring is considered to be the gold standard for diagnosing gastroesophageal reflux disease (GERD).⁵ Combined pH and impedance monitoring can detect all types of reflux episodes (acidic, weakly acidic, and alkaline) within the esophageal lumen and their composition (liquid, gas, or mixed). Moreover, 24-h multichannel intraluminal esophageal impedance or pH monitoring seems to be very promising for diagnosing pathological LPR.⁶

However, detecting pathological LPR can be difficult. Some episodes of LPR are present in healthy individuals; therefore, not every episode is pathological (causing extraesophageal reflux disease). There may be several episodes of reflux above the upper esophageal sphincter that do not harm the mucosa. Oelschlager et al. reported a median number of five pharyngeal reflux episodes in 10 asymptomatic controls during 24-h monitoring.⁷ On the other hand, Hoppo et al. more recently reported only one pharyngeal reflux event in 34 healthy subjects during 24-h monitoring.⁸ Zerbib et al. detected during 24-h monitoring a total of 32 pharyngeal reflux events in 12 healthy subjects with one subject having 12 pharyngeal reflux events. However, the median number of pharyngeal reflux events was zero.⁹

These discrepancies clearly demonstrate how the analysis of pharyngeal impedance tracings and diagnostics of LPR are challenging and require accurate and reproducible diagnostic criteria. The most accurate diagnostic method of relevant LPR remains to be pepsin detection in tissues.² However, this
requires a biopsy of the larynx, usually under general anesthesia that carries a risk of tissue scarring. Thus, it is not suitable for routine diagnostics, unlike pharyngeal impedance. There is also a new diagnostic method of LPR—salivary pepsin test, which is questioned for a routine diagnostic.\textsuperscript{10,11} Pepsin detection in saliva is much less invasive. On the contrary, there are several disadvantages. Pepsin positivity is measured in the oral cavity; therefore, a lot of pharyngeal refluxes, which reach only the hypopharynx, larynx, or oropharynx could be missed. Saliva samples must be obtained soon after a reflux event. Furthermore, even asymptomatic subjects have pepsin positivity in saliva and the cutoff for pathological LPR is missing. In the study of Yadlapati \textit{et al.}, neither oropharyngeal pH testing nor salivary pepsin analysis were able to distinguish between healthy volunteers and subjects with a combination of laryngeal and reflux symptoms.\textsuperscript{11}

Accordingly, the best way to improve routine LPR diagnostics is to improve the accuracy of pharyngeal impedance for the detection of relevant pathological LPR. Therefore, the aim of our study was to use pepsin detection in laryngeal mucosa for the identification of relevant pathological LPR and compare these findings to the results from 24-h pharyngoesophageal 8-channel intraluminal impedance/2-channel pH monitoring performed in the same patient 1 day before surgery to establish pharyngeal impedance values for pathological LPR.

\subsection*{METHODS}

This prospective study was approved by the Ethics Committee of the University Hospital Ostrava. It was performed in accordance with the Declaration of Helsinki and good clinical practice, and it followed the applicable regulatory requirements. The study was registered at ClinicalTrials.gov under the identifier: NCT02592902. Written informed consent was obtained from the patients before initiating any procedure. The study was conducted between January 2015 and December 2016. All authors had access to the study data and had reviewed and approved the final manuscript.

Adult patients with a diagnosed laryngeal pathology, in which a reflux might be considered as an etiopathogenetic factor such as vocal cord granuloma, recurrent laryngeal papillomatosis, and vocal cord polyp, who were referred for microlaryngoscopy were included in the study. Voice problems, breathing problems, and the necessity of histological verification of the pathology were the main indications for surgical treatment. All patients were off proton-pump inhibitor therapy. The exclusion criteria were patients with contraindications for general anesthesia, patients who did not consent to be included in the study, patients with a history of thoracic or digestive surgery (except appendectomy), gastrointestinal disease, consuming 40 g/day alcohol, smoking 20 cigarettes/day, and patients treated with medications that alter intragastric acidity or esophageal motility.

\textbf{24-h multichannel intraluminal impedance–dual-channel pH monitoring.} The 24-h multichannel intraluminal impedance–dual-channel pH monitoring, using the Digitrapper pH-Z Testing System (Medtronic, Minneapolis, MN, USA), was performed 1 day before surgery. A VersaFlex LPR ZNID19 +8R impedance catheter (Medtronic) was used with pH sensors at 0 cm (proximal) and 19 cm (distal), and eight impedance rings were located—1, 2, 5, 7, 9, 18, 20, and 22 cm from the proximal pH sensor (Figure 1). Before recording, the catheter was calibrated using buffer solutions at pH values of 4.0 and 7.0. The proximal pH sensor was placed in the hypopharynx 2 cm above the upper esophageal sphincter. The right sensor position was always verified using a flexible laryngoscope. A Digitrapper Recorder (Medtronic) was used for data recording. Patients were instructed to use the device to record the time they spent on eating, drinking, and in a horizontal position. Tracings were analyzed (after a visual check) using AccuView Reflux Software (Medtronic). The number of acid (pH < 4), weakly acid (pH 4–7), and alkaline pharyngeal reflux episodes were obtained.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Scheme of the VersaFlex\textsuperscript{\textregistered} LPR ZNID19+8R impedance catheter placement (green rectangle = impedance sensor, red circle = pH sensor).}
\end{figure}
**Pepsin detection.** Biopsy specimens of the laryngeal tissue were obtained during microlaryngoscopy procedures. Paraffin-embedded sections (2–3-μm thick) were prepared from the biopsy samples (Ventana Medical Systems, AZ, USA) and analyzed at the Department of Pathology by a single pathologist. Immunohistochemical analysis was performed after endogenous peroxidase blocking with hydrogen peroxide (Ventana) and antigen revitalization in CC1 buffer (Ventana). A pepsin antibody (NB100-66518, Novus Biologicals, CO, USA, diluted at a ratio of 1:100) was used as the primary antibody to detect pepsin. The incubation period for the primary antibody was 32 min. The iView DAB Detection Kit (Roche, Switzerland) was used to visualize the antigens. The presence of any antibody positivity in the cytoplasm of the cells was considered to be pathological and the sample was evaluated as pepsin positive.

**Statistical analysis.** The patients were divided into two groups, pepsin positive and pepsin negative. The results of the 24-h multichannel intraluminal impedance–dual-channel pH monitoring were compared between the groups. The number of LPR episodes in the pepsin-positive group was analyzed to establish a pathological cutoff value for LPR. Descriptive statistics, such as the arithmetic mean, standard deviation, and absolute and relative frequency tables, were used for data processing. The Shapiro–Wilk test was used to test for normality. The two-sample t-test, Mann–Whitney test, and Pearson’s χ² test were subsequently used based on the normality results. Fisher’s exact test was used when Pearson’s χ² test could not be used.

The calculation of sensitivity, specificity, and negative and positive predictive values with likelihood ratios were used for evaluation of the reliability of pepsin detection. Kaplan–Meier survival estimate, CHAID (Chi-square Automatic Interaction Detector), and Youden’s index were used as well. Receiver-operating curve was used for data visualization. The statistical tests were assessed using a significance level of 5%. The statistical analysis was performed using Stata 13 software (StataCorp., College Station, TX, USA). Risk groups identification was performed using SPSS Answer Tree 3.1 (IBM Corp., Armonk, NY, USA).

**RESULTS**

The study was conducted between January 2015 and December 2016. A total of 30 adult patients were included in the prospective study. Sixteen patients had vocal cord granuloma, 8 patients suffered from recurrent laryngeal papillomatosis, and 6 patients had vocal cord polyp. The average age of the participants was 43.7 ± 15.66 years. There were more men (53.3%) than women (46.7%).

**Groups.** Participants were divided into two groups according to the pepsin negativity/positivity determined from the laryngeal biopsy. There were 18 participants in the pepsin-negative group and 12 in the pepsin-positive group. There were no differences between the groups in terms of age, bodyweight, BMI, sex, history of immunodeficiency, allergy, diabetes mellitus, or tobacco exposure (Tables 1 and 2). There were more women in the pepsin-negative group (11/18; 61%) than in the pepsin-positive group (3/12; 25%), but this difference was not significant (P = 0.072).

**Comparison of 24-h pharyngoesophageal intraluminal impedance–pH monitoring results with pepsin detection–pharyngeal sensor.** The median of all pharyngeal reflux episodes detected by intraluminal impedance–pH monitoring was two in the pepsin-negative group and 14 in the pepsin-positive group. The range of pharyngeal reflux episodes varied from 0 to 5 in the pepsin-negative group and from 6 to 39 in the pepsin-positive group (P < 0.001) (Figure 2, Table 3). There was a significant difference in the number of all types of refluxes. Six or more pharyngeal reflux episodes were identified as the cutoff for the presence of pepsin in the laryngeal mucosa and, thereby, for the diagnosis of relevant/pathological LPR (Figures 3 and 4). The sensitivity for identifying pepsin (and diagnosing relevant/pathological LPR) using six or more pharyngeal refluxes as the cutoff was 100% (confidence interval (CI): 73.5–100%) and the specificity was 100% (CI: 81.4–100%). The negative and positive predictive values were 100%.

**Comparison of 24-h pharyngoesophageal intraluminal impedance–pH monitoring results with pepsin detection–esophageal sensor.** The median of all esophageal reflux episodes detected by intraluminal impedance–pH monitoring was two in the pepsin-negative group and 14 in the pepsin-positive group. The range of esophageal reflux episodes varied from 0 to 5 in the pepsin-negative group and from 6 to 39 in the pepsin-positive group (P < 0.001). The sensitivity for identifying pepsin (and diagnosing relevant/pathological LPR) using six or more esophageal refluxes as the cutoff was 100% (confidence interval (CI): 73.5–100%) and the specificity was 100% (CI: 81.4–100%). The negative and positive predictive values were 100%.

| Table 1 | General characteristics of the study participants according to pepsin negativity/positivity |
|---------|------------------------------------------------------------------------------------------|
| Pepsin  | No. | Median | Mean | SD | Min | Max | P value |
| Age     |     |        |      |    |     |     |         |
| Neg.    | 18  | 41.0   | 44.3 | 16.09 | 20 | 72 | 0.8022* |
| Pos.    | 12  | 39.5   | 42.8 | 15.64 | 19 | 74 |         |
| Height  |     |        |      |    |     |     |         |
| Neg.    | 18  | 169.0  | 170.9 | 12.38 | 152 | 195 | 0.0901* |
| Pos.    | 12  | 178.0  | 178.1 | 8.43 | 164 | 192 |         |
| Bodyweight |    |        |      |    |     |     |         |
| Neg.    | 18  | 81.0   | 82.7 | 19.95 | 57 | 114 | 0.2068* |
| Pos.    | 12  | 94.0   | 91.4 | 15.00 | 60 | 112 |         |
| BMI     |     |        |      |    |     |     |         |
| Neg.    | 18  | 27.8   | 28.2 | 5.43 | 21.5 | 43.7 | 0.4981* |
| Pos.    | 12  | 30.1   | 28.5 | 3.78 | 22.3 | 32.9 |         |

BMI, body mass index; SD, standard deviation.  
*Two-sample t-test.  
*Mann–Whitney test.

| Table 2 | Medical history of the study participants according to pepsin negativity/positivity |
|---------|--------------------------------------------------------------------------------|
| Pepsin  | Negative | Positive | P value* |
|         | Yes | No | Yes | No |
| Allergy | 2  | 16 | 3  | 9  | 0.364 |
| Tobacco exposure | 8  | 10 | 3  | 9  | 0.442 |
| Immunodeficiency | 0  | 18 | 0  | 18 | —    |
| Diabetes mellitus | 1  | 17 | 1  | 11 | 1.000 |

*Fisher’s exact test.
monitoring was 9 in the pepsin-negative group and 25 in the pepsin-positive group. The range of esophageal reflux episodes varied from 2 to 23 in the pepsin-negative group and from 8 to 58 in the pepsin-positive group ($P < 0.001$) (Table 4). There was a significant difference in the number of acid and alkaline refluxes. The esophageal acid exposure time and DeMeester Score were significantly higher in the pepsin-positive group (Table 4).

Sixteen or more esophageal reflux episodes were identified as the cutoff for the presence of pepsin in the laryngeal mucosa and, thereby, for the diagnosis of relevant/pathological LPR (Figure 5). The sensitivity for identifying pepsin using 16 or more esophageal refluxes as the cutoff was 83.3% (CI: 51.6–97.9%) and the specificity was 88.9% (CI: 65.3–98.6%). The negative and positive predictive values were 88.9% and 83.3%, respectively.

GERD was diagnosed if the number and type of the esophageal episode or DeMeester Score were pathological. There was one patient (5.6%) suffering from GERD in the pepsin-negative group and seven patients (58.3%) in the pepsin-positive group ($P = 0.001$, Pearson's $\chi^2$).

**DISCUSSION**

The aim of our study was to establish pathological values from 24-h pharyngoesophageal impedance/pH monitoring for the diagnosis of LPR. Pathological LPR was defined as the presence of pepsin in the cytoplasm of the laryngeal mucosa cells.

The advantage of pepsin detection over pharyngeal or esophageal impedance is that pepsin can be detected in

---

**Table 3** Comparison of the type and number of pharyngeal refluxes in patients in the pepsin-negative/positive groups

| Pharyngeal pH-metry results | Pepsin | Median | Mean | SD | Min | Max | $P$ value$^a$ |
|-----------------------------|--------|--------|------|----|-----|-----|-------------|
| Acidic reflux events        | Neg.   | 1.0    | 0.8  | 0.86 | 0   | 2   | <0.001      |
|                             | Pos.   | 7.0    | 8.0  | 5.62 | 0   | 20  |             |
| Weakly acidic reflux events | Neg.   | 1.0    | 1.6  | 1.42 | 0   | 4   | 0.0216      |
|                             | Pos.   | 4.0    | 7.6  | 9.73 | 0   | 35  |             |
| Alkaline reflux events      | Neg.   | 0.0    | 0.0  | 0.00 | 0   | 0   | 0.0281      |
|                             | Pos.   | 0.0    | 0.5  | 1.17 | 0   | 4   |             |
| Total reflux events         | Neg.   | 2.0    | 2.4  | 1.69 | 0   | 5   | <0.001      |
|                             | Pos.   | 13.5   | 16.0 | 10.39| 6   | 39  |             |

SD, standard deviation.

$^a$Mann–Whitney test.
tissues and fluids even when reflux has not occurred in the previous several days. Thus, it reflects a long-term situation. Pepsin is also present in all types of LPR (liquid, gas, or mixed) and is detected right in the examined tissue. Furthermore, the presence of pepsin in gastric refluxate is the main pathogenetic factor that causes proteolysis and cell damage. It is important to emphasize that pepsin was detected in the cytoplasm of laryngeal mucosa cells. For pepsin to be present in the cytoplasm, the reflux has to actually reach the larynx and overcome all the cell’s protecting mechanisms. Thus, if the cell’s cytoplasm is positive for pepsin, it has already damaged the cell and the LPR can be considered to be pathological. Therefore, the presence of pepsin was diagnosed using immunohistochemical analysis. Although it was not possible to establish pepsin concentration values, analysis enables a more precise evaluation of the samples. Other detection methods could provide pepsin concentration values; however, a sample is evaluated by these methods as a complex, and it cannot be established if the sample’s pepsin positivity is because of its extracellular or intracellular presence. Small extracellular pepsin concentrations could be normal in healthy individuals. On the other hand, every pepsin presence in the cell cytoplasm is pathological.

The results of pepsin detection were compared with the results from 24-h pharyngoesophageal 8-channel intraluminal impedance/2-channel pH monitoring performed in the same patient 1 day before surgery to establish pathological pharyngeal impedance values for LPR. A special impedance catheter for LPR diagnostics was used. The main disadvantage of the catheter is that GERD diagnostics can be difficult. Distal sensors are several centimeters higher in the esophagus than those in a standard esophageal impedance. Therefore, some minor esophageal refluxes that do not reach these slightly higher placed sensors might be missed. On the other hand, LPR diagnostics using this catheter is very precise. Thus, it was almost ideal for our study. Its exact placement was verified using flexible endoscopy in every case.

The participants were divided into two groups according to pepsin positivity/negativity in the laryngeal mucosa. The groups were homogeneous in terms of age, bodyweight, BMI, sex, history of immunodeficiency, allergy, diabetes mellitus, or tobacco exposure. Patients who were positive for pepsin in the laryngeal mucosa were considered to have pathological LPR.

According to the results of our study and previous data, the type of LPR does not play such an important role in its pathogenicity. There was a significant difference in the number of all types of refluxes in the study. It is likely that pepsin is the main pathogenetic factor present in every type of LPR. It is well known that pepsin is inactive but stable at a pH of 7.0 (alkaline pharyngeal reflux), and that it can be reactivated upon reacidification, retaining 79 ± 11% of its original activity at a pH of 3.0. The reacidification could happen anytime due to acid reflux or simply by eating acidic food.

The total number of all pharyngeal reflux episodes seems to be the most crucial factor for relevant LPR identification. Pharyngeal reflux episodes were relatively rare events in the pepsin-negative group with a median of two episodes. This finding is in agreement with previous studies in asymptomatic patients. However, there were reports of up to five episodes. The median number of pharyngeal reflux episodes

Table 4 Comparison of the type and number of esophageal refluxes in patients in the pepsin-negative/positive groups

| Esophageal pH-metry results | Pepsin | Median | Mean | SD  | Min | Max | P value* |
|----------------------------|--------|--------|------|-----|-----|-----|---------|
| Acidic reflux events       | Neg.   | 2.0    | 2.3  | 2.00| 0   | 8   | 0.0004  |
|                           | Pos.   | 11.5   | 14.3 | 10.66| 0   | 37  | 0.1742  |
| Weakly acidic reflux events| Neg.   | 7.5    | 7.5  | 5.08| 1   | 20  | 0.0281  |
|                           | Pos.   | 9.0    | 12.8 | 11.98| 2   | 46  | 0.0005  |
| Alkaline reflux events     | Neg.   | 0.0    | 0.0  | 0.00| 0   | 0   | 0.0002  |
|                           | Pos.   | 0.0    | 0.5  | 1.17| 0   | 4   |         |
| Total reflux events        | Neg.   | 8.5    | 9.8  | 5.77| 2   | 23  |         |
|                           | Pos.   | 25.0   | 27.7 | 15.33| 8   | 58  |         |
| Esophageal acid exposure time (min) | Neg. | 9.5    | 12.1 | 11.19| 1   | 43  |         |
|                           | Pos.   | 52.5   | 56.4 | 38.67| 9   | 111 |         |
| DeMeester score            | Neg.   | 4.6    | 5.1  | 4.02| 0.3 | 16.7| 0.0015  |
|                           | Pos.   | 19.1   | 19.6 | 13.55| 2.9 | 42.3|         |

SD, standard deviation.
*Mann–Whitney test.

Figure 5 Receiver-operating curve showing the sensitivity and specificity for identifying pepsin in laryngeal mucosa considering the total esophageal refluxes.
in the pepsin-positive group was 14 (range 6–39). Statistical analysis confirmed that six or more pharyngeal reflux episodes was the cutoff value for pepsin in the laryngeal mucosa and, thereby, for the diagnosis of relevant LPR affecting the larynx. The sensitivity and specificity for the pathological LPR diagnosis reached 100%. However, the CIs were 73.5–100% and 81.4–100%, respectively. This was due to the small cohort, which is also the main limitation of our study. On the contrary, statistical power for pharyngeal reflux test is 100%. A clear-cut result in the case of pharyngeal reflux is surprising, and the authors suppose that with a growing cohort, the cutoff becomes much less clear.

Data from a slightly higher placed distal sensor are not absolutely comparable understanding that the technique is not optimized for GER evaluation. Statistical analysis revealed that 16 esophageal episodes could be the cut point value for the diagnosis of relevant LPR affecting the larynx. The sensitivity and specificity is lower than that in the case of the detection of pharyngeal reflux episodes. Patients with a higher esophageal acid exposure time and DeMeester Score were more likely to represent pathologic LPR.

Six pharyngeal reflux episodes registered during 24-h impedance–pH monitoring seem to be the cutoff for diagnosing pathological LPR. Therefore, it is possible to suggest establishing of this value as the pathological 24-h impedance value indicating pathological LPR. This result is based on a comparison with pepsin detection in laryngeal mucosa, which is the most accurate way to diagnose LPR. These results must be interpreted with caution, and additional studies with larger cohorts are warranted to confirm these findings.

CONFLICT OF INTEREST

Guarantor of the article: Pavel Komínek, MD, PhD, MBA.
Specific author contributions: Martin Formánek—study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; statistical analysis. Debora Jančářová—study concept and design; acquisition of data; analysis and interpretation of data. Pavel Komínek—critical revision of the manuscript for important intellectual content; study supervision. Radoslava Tomanová—acquisition of data. Karol Zeleník—study concept and design; analysis and interpretation of data; and critical revision of the manuscript for important intellectual content.

Financial support: This research was supported by the Ministry of Health, Czech Republic—conceptual development of research organization (FNOS/2014).
Potential competing interests: None.

Acknowledgments. The authors thank Hana Tomášková, PhD, for her help with statistical analysis.