

Association of Salivary Pepsin with Systemic Inflammatory Biomarkers and Laryngeal Disease Severity in Patients with Laryngopharyngeal Reflux–Associated Chronic Laryngitis

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Abstract

Background: Laryngopharyngeal reflux (LPR) is clinically heterogeneous and symptoms and/or laryngoscopic signs alone have limited specificity. While salivary pepsin is a non-invasive measure of gastric-content exposure, its association with systemic inflammation and severity of laryngeal disease is not well described. **Objectives:** to assess the correlation between salivary pepsin concentration and the systemic inflammatory biomarkers in chronic laryngitis associated with LPR. **Materials and Methods:** This was a prospective analytical comparative case-control study involving 180 adults: 60 patients with LPR-associated chronic laryngitis, 60 patients with chronic laryngitis without objective evidence of LPR and 60 healthy controls. Three timed saliva samples were analysed for pepsin A using quantitative enzyme-linked immunosorbent assay (ELISA). High sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α), complete blood count-derived indices, Reflux Symptom Score-12 (RSS-12) and Reflux Sign Assessment-10 (RSA-10) were assessed. Multivariable models and receiver operating characteristic (ROC) analyses were used. **Results:** Maximum salivary pepsin was highest in the LPR group (median, 84.6 ng/mL; interquartile range [IQR], 57.3–126.8), followed by non-LPR chronic laryngitis (24.8 ng/mL; IQR, 13.1–43.7) and controls (8.7 ng/mL; IQR, 4.2–17.6; $p < 0.001$). The graded pattern was also observed for IL-6, hs-CRP, TNF- α , NLR, PLR, and SII. Pepsin correlated with IL-6 ($r_s = 0.54$), hs-CRP ($r_s = 0.47$), RSS-12 ($r_s = 0.63$), and RSA-10 ($r_s = 0.59$; all $p < 0.001$). In the comparison with non-LPR chronic laryngitis, pepsin (adjusted odds ratio [aOR] per 10 ng/mL, 1.28; 95% confidence interval [CI], 1.14–1.44) and IL-6 (aOR per 1 pg/mL, 1.31; 95% CI, 1.09–1.58) were independently associated with LPR. The area under the ROC curve (AUC) for pepsin alone was 0.874 and the AUC for the combined biomarker-clinical model was 0.944 (internally validated). **Conclusions:** Quantitative salivary pepsin correlated with systemic inflammatory activation and severity of symptoms and laryngoscopy. Addition of pepsin to IL-6 and hs-CRP and clinical covariates increased discrimination between LPR-associated and non-LPR chronic laryngitis.

Keywords: Chronic laryngitis, Interleukin-6, Laryngopharyngeal reflux, Pepsin, Saliva, Systemic inflammation.

Introduction

Laryngopharyngeal reflux (LPR) is defined as exposure of the laryngopharyngeal mucosa to gastroduodenal refluxate and the resulting morphological, inflammatory and/or neurological changes. Dysphonia (hoarseness), throat clearing, globus sensation, chronic cough, excessive mucus and throat discomfort are common complaints, and often, there is no

typical heartburn. These presentations are similar to those seen in allergy, infection, vocal overuse, smoking related irritation, and functional laryngeal disorders, and as such, the diagnosis is always uncertain based on symptoms alone [1,2]. The modern approach to diagnosis is thus to phenotype and objectively assess reflux rather than to empirically suppress acid for a long period of time. The International Federation of

Oto-Rhino-Laryngological Societies (IFOS) Dubai consensus defined LPR as an upper aerodigestive tract disease related to direct and/or indirect effects of gastroduodenal reflux and proposed that the presence of more than one acid, weakly acid or nonacid hypopharyngeal reflux event during 24-hour hypopharyngeal-esophageal multichannel intraluminal impedance pH (HEMII-pH) monitoring can support the diagnosis [3]. This is crucial as the signs on laryngoscopy are not pathognomonic and conventional esophageal testing may not be able to record events in the proximal esophagus well. Normally, pepsin is not synthesized by the tissues of the laryngopharynx and is synthesized in the stomach. Thus, its presence in saliva is an indicator of the presence of gastric material in the upper aerodigestive tract. Pepsin can reactivate in the epithelial cells after internalization by exposure to low pH, resulting in an increase in epithelial permeability, a shift in stress response and inflammatory signalling. Experimental studies suggest that nuclear factor-kappa B, inflammasome activation, and cytokines like IL-1 β , IL-6 and IL-8 are involved pathways [4,5]. In general, clinical studies have found that the salivary levels of pepsin are higher in LPR than in asymptomatic controls, but with varying levels of sensitivity and specificity depending on the assay platform, cutoff, sampling site and timing. A systematic review revealed that the diagnostic performance of the studies was quite variable and generally low, while recent timed-sampling studies demonstrated that fasting samples and bedtime samples are complementary [6,7]. These results support multiple quantitative sampling, as opposed to a single dichotomous test. There could be local reflux related inflammation in the setting of a detectable systemic inflammatory activation. C-reactive protein (hs-CRP), interleukin-6 (IL-6) and tumour necrosis factor

alpha (TNF- α) are indicators of different aspects of inflammation. Neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) are among the easily obtained ratios that reflect the innate immune and thrombo-inflammatory balance and are inexpensive [8]. However, the mutual interdependency of quantitative salivary pepsin, blood cytokines, CBC-based indices and standardized laryngeal severity has been little studied directly in chronic laryngitis in relation to LPR [9]. The main objective was to assess the correlation between salivary pepsin concentration and the systemic inflammatory biomarkers in chronic laryngitis associated with LPR. Secondary objectives were to assess differences between the biomarkers of each of the three groups (LPR associated chronic laryngitis, non-LPR chronic laryngitis, and healthy controls), assess the association of the biomarkers with RSS-12 and RSA-10, identify independent biomarkers associated with LPR-associated disease, and determine the diagnostic accuracy of the individual and combined biomarker models. We speculated that salivary pepsin levels would be directly linked to the extent of inflammation in the system and to the severity of laryngeal disease.

Materials and Methods

Study design and setting

The study was a prospective analytical comparative case-control study that was carried out in the otolaryngology outpatient and laryngology services in Al-Hilla Surgical Teaching Hospital and Imam Al-Sadiq Teaching Hospital in Babel Province, Iraq, between January and June 2026. Objective reflux monitoring was coordinated with a participating gastroenterology unit that had 24-hour hypopharyngeal-esophageal multichannel intraluminal impedance-pH monitoring. The

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laboratory analyses were carried out in specialized private laboratories with standardized platforms, which are accredited in Hilla. Reporting followed STROBE. There was consecutive recruitment for the symptomatic and healthy controls were frequency matched by age and sex.

Participants and groups

Adults (18-65 years old) were recruited into three equally sized groups. Group I included patients with chronic laryngeal symptoms for >12 weeks, videolaryngoscopic findings of chronic inflammation and objective evidence of LPR on 24-hour HEMII-pH monitoring. Group II included patients with chronic laryngitis, but who did not have a qualifying hypopharyngeal reflux event on HEMII-pH. Group III consisted of healthy volunteers who did not have persistent laryngeal symptoms, clinically significant laryngoscopic changes, or have received treatment for reflux in the past six months. Salivary pepsin results were not used to define group membership, thus eliminating incorporation bias.

Eligibility criteria

The symptomatic eligible participants had dysphonia, throat clearing, globus, cough, mucus sensation, or throat irritation as their primary symptom and chronic laryngeal inflammation. Exclusion criteria included the presence of an acute respiratory infection or an acute laryngitis in the 4 weeks prior to the study; known autoimmune or systemic inflammatory disease, active malignancy, previous laryngeal cancer or recent surgery, major structural vocal-fold lesions unrelated to reflux, current immunosuppressive therapy or use of systemic corticosteroids, severe uncontrolled chronic disease, pregnancy, and inability to complete the protocol. Universal exclusions were not applied

to current smoking and relevant comorbidities, which were measured and adjusted. When clinically safe, participants discontinued proton-pump inhibitors 7 days prior to reflux monitoring, H₂-receptor antagonists 72 hours prior to reflux monitoring, and alginates/antacids 24 hours prior to reflux monitoring.

Sample-size determination

The planned sample of 180 (60 per group) yielded at least 80% power at a two-sided α of 0.05 to detect a standardized between-group difference of approximately 0.50 allowing for three group comparisons and incomplete samples. Model complexity was controlled through prespecified predictors and penalized sensitivity analysis for logistic regression for the two chronic-laryngitis groups (60 events and 60 non-events). The sample size was also sufficient to provide a reasonably precise estimation of an AUC of approximately 0.85.

Clinical assessment

Age, sex, body mass index (BMI), smoking, occupational voice use, coffee consumption, late night eating, duration of symptoms, typical gastroesophageal reflux symptoms, previous reflux therapy, diabetes, hypertension, asthma, allergic rhinitis and medication were collected on a standardized form. BMI was defined as weight (kg) divided by height (m²).

Symptom and laryngoscopic assessment of the vocal folds.

Participants filled out the Reflux Symptom Score-12 (RSS-12), a validated patient-reported outcome instrument which assesses the frequency, severity and impact of LPR symptoms [10]. A standardized protocol of flexible video-laryngoscopy was performed. Recordings were independently double-scored by two ENT physicians (blinded to the

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laboratory results and group allocation) using the Reflux Sign Assessment-10 (RSA-10) which quantifies the common oral, pharyngeal and laryngeal findings [11]. The scores from the reviewers were averaged [12]. A two-way random-effects intraclass correlation coefficient (ICC) was used to quantify inter-rater reliability [13].

Objective reflux assessment

HEMII-pH monitoring was done following an overnight fast with the proximal impedance segment placed across the upper esophageal sphincter as per manufacturer's instructions. Meals, supine time and symptoms documented. Tracings were independently reviewed by trained investigators. LPR was accompanied by >1 acid, weakly acid or nonacid hypopharyngeal reflux event in 24 hours, consistent with the Dubai consensus [3]. The consensus method was used to resolve discordant readings.

Collection of saliva and pepsin assay

The three saliva samples were taken at three different times in the day, without any stimulation: 1) immediately after waking up and before breakfast, 2) 60 minutes after the standardized main meal, and 3) at bedtime, and placed in separate sterile tubes. Food and other liquids (except water), tooth brushing, smoking, and vigorous exercise were abstained from for at least 60 minutes prior to collection. Samples were put on ice, centrifuged at $3,000 \times g$ for 10 minutes within one hour, aliquoted and stored at -80°C without repeated freeze-thaw cycles. Pepsin A was measured in duplicate using human pepsin ELISA kit (validated for saliva) according to the manufacturer's instructions. Laboratory staff was masked with respect to clinical group. Samples with concentrations that fell outside of the calibration range were re-assayed at an appropriate dilution. Repeat

analysis was performed if the CV was greater than 15%. The highest of the three timed concentrations was the primary salivary exposure and the mean concentration and number of samples positive at exploratory cutoff of 50 ng/mL were secondary measures.

Blood biomarkers

Venous blood was taken from the arm after an overnight fast (08:00-10:00) on the day of clinical assessment. Immunoturbidimetry method was used to determine serum hs-CRP. High sensitivity ELISA kits were used to quantify IL-6 and TNF- α , in duplicate. The same analyzer was used for a complete blood count (CBC) including differential. NLR was defined as the absolute neutrophils count/absolute lymphocytes count, PLR was determined as platelets count/absolute lymphocytes count, and SII was determined as platelets count $\times$ absolute neutrophils count/absolute lymphocytes count. The units were standardized prior to calculating.

Bias control and outcomes

The main result was the relationship between the highest salivary pepsin level and systemic inflammatory markers in all subjects and in subjects with chronic-laryngitis. Secondary outcomes were group differences, relationship with RSS-12/RSA-10, independent predictors of LPR-associated chronic laryngitis and diagnostic discrimination. Bias control was done by consecutive recruitment, objective group assignment (not pepsin-dependent), standardized timed sampling, blinded duplicate assays, blinded dual laryngoscopic scoring, prespecified confounders, and complete-case and multiple-imputation sensitivity analysis.

Statistical analysis

Data analyses were carried out in R 4.4 or SPSS 29. Histograms, Q-Q plots and Shapiro-Wilk

tests were used to evaluate distribution. Normally distributed variables are expressed as mean $\pm$ standard deviation and analyzed by one-way analysis of variance (ANOVA), Tukey correction. Skewed variables are reported as median (IQR) and the Kruskal–Wallis test with Dunn–Holm post hoc comparisons are used for comparison. Categorical data are reported as numbers (percentages) and analyzed using chi-square or Fisher exact test. Effect sizes are presented as standardized mean differences, rank-biserial, Cramér's V, odds ratios or regression coefficients with 95% CIs as appropriate. The relationships between the biomarkers were evaluated using Spearman correlations. Using multivariable logistic regression, LPR-associated chronic laryngitis was compared to non-LPR chronic laryngitis. Age, sex, BMI, smoking status, duration of the symptoms, pepsin, IL-6, hs-CRP and SII were prespecified as covariates and collinearity was tested by variance inflation factors. A multivariable linear model was used to assess the severity of the RSA-10. Restricted cubic splines were used to check for non-linearity, along with residual diagnostics. ROC curves were created for the individual biomarkers and sequential models. The optimal cutoffs were obtained using the Youden index, and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Optimism was estimated using 1,000 bootstrap re-sample. Two-sided p-values of <0.05 were considered significant for secondary biomarkers where false-discovery rate (FDR) was controlled using Benjamini–Hochberg FDR.

Ethical considerations

Scientific and ethical clearance for the protocol was obtained from the appropriate institutional research ethics committee, and administrative permission from the hospitals involved, prior to

recruitment. Written informed consent was obtained from all subjects. Studies complied with Declaration of Helsinki. Records were coded and held on password protected records and only accessed by the research team. Appropriate communication of clinically important findings was made and referrals were made for appropriate care.

Operational definitions and diagnostic adjudication:

Chronic laryngitis was considered as the presence of laryngeal symptoms for a period of at least 12 weeks and inflammatory findings on the videolaryngoscopy. Weakly acid and nonacid hypopharyngeal reflux events in 24-hour HEMII-pH monitoring were also considered as objective LPR, if there was more than one occurrence. No attempt was made to allocate subjects to diagnostic groups with pepsin. Prior to adjudication, acute infection, structural disease of the vocal folds, allergy-dominant symptoms, smoking exposure, asthma, occupational voice use and pertinent medications were documented. The two investigators analyzed HEMII-pH tracings independently; in cases of disagreement the result was determined by consensus prior to the release of biomarker results.

Handling of specimens and quality assurance in the laboratory

Saliva was collected in pre-labeled sterile tubes and transferred immediately on wet ice and centrifuged within one hour, aliquoted for single use and stored at -80°C ; freeze – thaw cycles were not allowed. Blood samples were taken under uniform time of day and fasting conditions. All ELISA measurements were conducted within the same validated platform, using the same lot of reagents whenever possible, calibration standards and manufacturer controls on each plate, placement of samples in a

random manner and prespecified acceptance limits. Repeat testing was done at an appropriate dilution when duplicate CVs were >15%, controls failed or were outside of the analytic range. The laboratory audit file contained the lot numbers, calibration curves, lower limits of detection and intra/inter-assay coefficients of variation.

Managing data, missing data and reproducibility

Variable names, units, ranges of values, timing windows and derived indices were defined by a prespecified case-report form and data dictionary. Double data entry was performed and automated range, logic and duplication checks were performed prior to database lock. Labels of groups were blinded for the laboratory personnel and the biomarkers' values were blinded for clinical raters. Documentation of reasons for missingness. Multiple imputation was used in the primary analysis when the amount of missingness was low (below 10%) and the missingness in the predictor variables was plausibly missing at random, and complete-case sensitivity analysis was used. The analysis code, de-identified data template and final statistical-analysis plan were version controlled and stored for use in reproducibility.

Prespecified sensitivity analyses

Sensitivity analyses involved excluding current smokers and diabetes, excluding samples collected outside the required time window, excluding samples collected outside the required time window and using mean pepsin instead of maximum pepsin, excluding samples with active allergic rhinitis and excluding the samples separately for fasting, postprandial, and bedtime samples, and using penalized logistic regression to assess the small-sample bias. ROC cutoffs were used as exploratory and were not used in

clinical practice without external validation. Predictive values were considered with regard to the sampling fraction among the case-control population.

Results:

One hundred and ninety of 207 people evaluated were eligible and eight refused to participate. Finally, 180 participants were included with 60 in each group. Baseline comparability was achieved in terms of age, sex, BMI, smoking, diabetes, hypertension, asthma and allergic rhinitis which were not significantly different among the groups. LPR group had more common symptoms of reflux and late-night feeding. For 176 participants (97.8%) complete biomarker data was available; four cytokine values were multiple imputed, with negligible difference between complete case and multiple imputation estimates.

Table 1. Demographic and clinical characteristics of the study groups are shown.

Characteristic	LPR chronic laryngitis (n=60)	Non-LPR chronic laryngitis (n=60)	Healthy controls (n=60)	p value
Age, years	42.8 ± 10.6	41.5 ± 11.2	40.9 ± 10.1	0.624
Female sex	34 (56.7)	33 (55.0)	35 (58.3)	0.934
BMI, kg/m ²	27.6 ± 4.1	26.9 ± 4.3	26.5 ± 3.9	0.322
Current smoking	11 (18.3)	12 (20.0)	9 (15.0)	0.763
Diabetes mellitus	7 (11.7)	6 (10.0)	5 (8.3)	0.829
Allergic rhinitis	12 (20.0)	14 (23.3)	9 (15.0)	0.504
Late-night meals ≥3/week	38 (63.3)	25 (41.7)	17 (28.3)	<0.001
Typical reflux symptoms	36 (60.0)	18 (30.0)	5 (8.3)	<0.001

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Symptom duration, months	14 (8–24)	12 (7–22)	—	0.348
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Values are expressed as mean $\pm$ SD, median (IQR) or n. BMI, body mass index; LPR, laryngopharyngeal reflux.

The RSS-12 and RSA-10 scores were the highest in chronic laryngitis due to LPR. Throat clearing, excessive mucous production, globus and dysphonia occurred more often in both groups with chronic laryngitis than in LPR, and in both groups were common. Other features that were enriched in the LPR group included posterior commissure hypertrophy, diffuse endolaryngeal edema and interarytenoid erythema. There was excellent agreement between the two laryngoscopic reviewers (ICC=0.89; 95% CI, 0.85–0.92).

Table 2. Symptom scores and laryngoscopic findings are used to assess the severity of LPR.

Measure	LPR (n=60)	Non-LPR (n=60)	Controls (n=60)	p value
RSS-12 total	44.2 $\pm$ 13.1	27.6 $\pm$ 11.4	5.8 $\pm$ 4.6	<0.001
RSA-10 total	24.7 $\pm$ 6.8	15.2 $\pm$ 5.9	4.1 $\pm$ 2.8	<0.001
Throat clearing	49 (81.7)	37 (61.7)	6 (10.0)	<0.001
Globus sensation	41 (68.3)	26 (43.3)	3 (5.0)	<0.001
Dysphonia	45 (75.0)	39 (65.0)	4 (6.7)	<0.001
Posterior commissure hypertrophy	43 (71.7)	28 (46.7)	5 (8.3)	<0.001
Interarytenoid erythema	47 (78.3)	35 (58.3)	8 (13.3)	<0.001
Thick endolaryngeal mucus	39 (65.0)	23 (38.3)	4 (6.7)	<0.001

All data are mean $\pm$ SD or number (n); RSA-10, Reflux Sign Assessment-10; RSS-12, Reflux Symptom Score-12.

The salivary pepsin levels showed a graded group pattern for maximum, mean and time specific salivary pepsin levels. Median concentration was the highest in the LPR group in postprandial samples and there was a further group of positive individuals identified in the

bedtime sample. Eighty percent of the LPR, 20 percent of the chronic laryngitis (non-LPR) and 5 percent of the controls had at least one sample at or above 50 ng/mL ($p < 0.001$). The results of all pairwise comparisons for maximum and mean concentration were still significant after Holm correction.

Table 3. The prevalence of positivity for salivary pepsin and its concentration.

Pepsin measure	LPR (n=60)	Non-LPR (n=60)	Controls (n=60)	p value
Fasting, ng/mL	43.8 (25.4–70.1)	14.7 (7.8–27.5)	5.2 (2.7–10.6)	<0.001
Postprandial, ng/mL	68.5 (42.1–103.7)	19.3 (9.9–35.4)	6.9 (3.1–14.5)	<0.001
Bedtime, ng/mL	54.2 (31.0–88.9)	16.1 (8.3–30.7)	5.7 (2.8–12.3)	<0.001
Maximum, ng/mL	84.6 (57.3–126.8)	24.8 (13.1–43.7)	8.7 (4.2–17.6)	<0.001
Mean, ng/mL	55.5 (35.2–82.4)	17.2 (9.4–30.1)	6.2 (3.0–12.1)	<0.001
≥ 1 sample ≥ 50 ng/mL	48 (80.0)	12 (20.0)	3 (5.0)	<0.001
≥ 2 samples ≥ 50 ng/mL	34 (56.7)	4 (6.7)	0 (0.0)	<0.001

The concentrations were median (IQR) and the number of positives is n; the overall comparisons were made using Kruskal–Wallis or chi-square tests.

hs-CRP, IL-6 and TNF- α levels were significantly higher in the LPR group compared to both comparison groups. Chronic laryngitis alone (non-LPR) had smaller but significant differences compared with controls, indicating inflammation associated with chronic laryngitis. All three biomarkers had q values of < 0.01 .

Table 4. Circulating inflammatory biomarkers

Biomarker	LPR (n=60)	Non-LPR (n=60)	Controls (n=60)	p value	FDR q
hs-CRP, mg/L	3.7 (2.2–5.8)	2.1 (1.2–3.4)	1.1 (0.6–1.9)	<0.001	<0.001
IL-6, pg/mL	6.8 (4.7–9.6)	4.1 (2.8–5.9)	2.3 (1.5–3.4)	<0.001	<0.001
TNF- α , pg/mL	12.9 (9.8–17.1)	9.4 (7.1–12.8)	6.8 (5.2–9.0)	<0.001	<0.001

Data are median (IQR). FDR, false-discovery rate; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; TNF- α , tumour necrosis factor-alpha.

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Neutrophils and platelets were slightly increased and lymphocytes slightly decreased in disease associated with LPR. Therefore, highest values of NLR, PLR and SII were observed in LPR group. This was the same when smokers and diabetics were excluded.

Table 5. The complete blood count (CBC) contains parameters, such as indices that are derived from the data.

Measure	LPR (n=60)	Non-LPR (n=60)	Controls (n=60)	p value
Neutrophils, $\times 10^9/L$	4.48 $\pm$ 1.12	4.02 $\pm$ 1.05	3.68 $\pm$ 0.91	<0.001
Lymphocytes, $\times 10^9/L$	1.89 $\pm$ 0.48	2.03 $\pm$ 0.51	2.18 $\pm$ 0.49	0.004
Platelets, $\times 10^9/L$	286 $\pm$ 61	267 $\pm$ 58	252 $\pm$ 54	0.006
NLR	2.43 (1.91–3.05)	1.98 (1.56–2.52)	1.67 (1.34–2.12)	<0.001
PLR	151 (123–186)	132 (106–164)	116 (95–142)	<0.001
SII, $\times 10^9/L$	691 (512–914)	523 (389–698)	426 (318–562)	<0.001

Data presented as values are mean $\pm$ SD or median (IQR). NLR = neutrophil-to-lymphocyte ratio; PLR = platelet-to-lymphocyte ratio; SII = systemic immune-inflammation index.

There were moderate positive correlations between maximum salivary pepsin and IL-6, hs-CRP, TNF- α and SII and weak to moderate positive correlations between maximum salivary pepsin and NLR and PLR. Directionality of associations did not change within the 120 chronic-laryngitis patients. There was the closest clinical correlation with RSS-12 and RSA-10.

Table 6. Spearman correlations between maximum salivary pepsin and systemic inflammatory biomarkers Systemic inflammatory biomarkers (P<0.05)

Variable	All participants r_s	p value	Chronic laryngitis only r_s	p value
hs-CRP	0.47	<0.001	0.39	<0.001
IL-6	0.54	<0.001	0.46	<0.001
TNF- α	0.42	<0.001	0.34	<0.001
NLR	0.36	<0.001	0.29	0.001
PLR	0.31	<0.001	0.25	0.006

SII	0.40	<0.001	0.33	<0.001
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hs-CRP, high sensitivity C-reactive protein; IL-6, interleukin-6; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; SII, systemic immune-inflammation index; TNF- α , tumor necrosis factor alpha.

Table 7. Correlation of Biomarkers with the severity of symptoms and laryngoscopy

Biomarker	RSS-12 r_s	p value	RSA-10 r_s	p value
Maximum salivary pepsin	0.63	<0.001	0.59	<0.001
hs-CRP	0.42	<0.001	0.39	<0.001
IL-6	0.49	<0.001	0.46	<0.001
TNF- α	0.35	<0.001	0.33	<0.001
NLR	0.32	<0.001	0.30	<0.001
PLR	0.27	<0.001	0.25	0.001
SII	0.36	<0.001	0.34	<0.001

RSA-10 = Reflux Sign Assessment-10, RSS-12 = Reflux Symptom Score-12.

In the LPR group, the subjects were divided into three groups (mild, moderate, and severe) according to prespecified tertiles of the RSA-10. There was a monotonic increase with severity in pepsin and inflammatory biomarkers. The trend tests of pepsin, IL-6, hs-CRP and SII were significant.

Table 8. Biomarkers based on severity of the disease using the laryngoscopy in the LPR group.

Biomarker	Mild (n=20)	Moderate (n=20)	Severe (n=20)	p for trend
Maximum pepsin, ng/mL	55.4 (41.2–73.8)	82.7 (62.6–108.5)	132.1 (96.8–171.4)	<0.001
hs-CRP, mg/L	2.4 (1.6–3.5)	3.6 (2.4–5.1)	5.7 (3.8–7.6)	<0.001
IL-6, pg/mL	4.8 (3.7–6.1)	6.6 (5.1–8.2)	9.5 (7.2–12.4)	<0.001
TNF- α , pg/mL	10.4 (8.2–13.0)	12.7 (9.9–15.9)	16.8 (12.9–20.8)	0.002
SII, $\times 10^9/L$	552 (431–690)	682 (528–843)	866 (655–1084)	<0.001

Data are median (IQR). Severity categories were predefined by LPR group using the prespecified tertiles for the RSA-10.

After making adjustments, maximum salivary pepsin and IL-6 still proved to be independent risk factors for LPR in the chronic-laryngitis

comparison. Hs-CRP demonstrated a less independent association while SII was not significant after the addition of cytokines and pepsin. VIFs were less than 2.2. The results from a penalized logistic regression model were similar, with no influential observation changing conclusions.

Table 9. Multivariable predictors of LPR-associated chronic laryngitis in all of the symptomatic participants (n=120).

Predictor	Adjusted OR	95% CI	p value
Maximum pepsin, per 10 ng/mL	1.28	1.14–1.44	<0.001
IL-6, per 1 pg/mL	1.31	1.09–1.58	0.004
hs-CRP, per 1 mg/L	1.24	1.01–1.52	0.041
SII, per 100 units	1.08	0.94–1.24	0.278
BMI, per 1 kg/m ²	1.05	0.95–1.16	0.341
Current smoking	1.22	0.43–3.47	0.708
Age, per year	1.01	0.97–1.06	0.562
Female sex	1.09	0.47–2.54	0.840
Symptom duration, per 6 months	1.07	0.94–1.22	0.302

Outcome: Chronic laryngitis LPR associated vs. non-LPR. OR, odds ratio; CI, confidence interval. Model Nagelkerke R²= 0.58, Hosmer – Lemeshow p=0.61.

Maximum pepsin (standardized β =0.38; p<0.001), IL-6 (β =0.24; p=0.003) and hs-CRP (β =0.16; p=0.032) were independently associated with RSA-10 in the linear severity model after adjustment for age, sex, BMI, smoking and SII. The variance of the RSA-10 explained by the model was 49% (adjusted R² = 0.49). Maximum pepsin was the individual discriminator best for discriminating LPR-associated chronic laryngitis from non-LPR chronic laryngitis. The sensitivity and specificity for a cut-off of 48.5 ng/mL were 80.0% each. The discrimination of IL-6 was moderate, with indices derived from CBC being weaker. In the case-control sample, performance measures were calculated and should not be used as prevalence adjusted population predictive values.

Table 10. Single biomarkers for diagnostic performance of chronic laryngitis in LPR

Marker	AUC (95% CI)	Cutoff	Sensitivity %	Specificity %	PPV %	NPV %
Maximum pepsin	0.874 (0.808–0.940)	48.5 ng/mL	80.0	80.0	80.0	80.0
IL-6	0.766 (0.680–0.852)	5.15 pg/mL	71.7	70.0	70.5	71.2
hs-CRP	0.712 (0.620–0.804)	2.75 mg/L	66.7	68.3	67.8	67.2
TNF- α	0.694 (0.598–0.790)	10.8 pg/mL	65.0	66.7	66.1	65.6
NLR	0.681 (0.584–0.778)	2.18	63.3	65.0	64.4	63.9
PLR	0.644 (0.544–0.744)	143	58.3	63.3	61.4	60.3
SII	0.699 (0.604–0.794)	603	65.0	66.7	66.1	65.6

AUC: area under the curve; CI: confidence interval; NPV: negative predictive value; PPV: positive predictive value. PPV and NPV are based on the composition of the case-control group (1:1).

Table 11. Sequential models - diagnostic performance

Model	Predictors	Apparent AUC (95% CI)	Bootstrap-corrected AUC	Sensitivity %	Specificity %
Model 1	Maximum pepsin	0.874 (0.808–0.940)	0.868	80.0	80.0
Model 2	IL-6 + hs-CRP + SII	0.826 (0.750–0.902)	0.813	76.7	75.0
Model 3	Pepsin + IL-6 + hs-CRP	0.927 (0.881–0.973)	0.916	85.0	86.7
Model 4	Model 3 + age + sex + BMI + smoking + symptom duration	0.951 (0.915–0.987)	0.944	88.3	90.0

AUC: area under the receiver operating characteristic curve, BMI: body mass index. The number of bootstrap resamples used in Optimism correction was 1000.

Discussion

This comparative biomarker study revealed a logical continuum from objective LPR to salivary pepsin exposure to systemic

inflammation to the severity of the laryngeal disease. The concentration of maximum pepsin was about three times as high in chronic laryngitis of LPR patients compared to non-LPR chronic laryngitis and almost ten times as high compared to healthy controls. IL-6, hs-CRP, TNF- α , NLR, PLR and SII had the same ranked order. Pepsin was found to be associated with systemic inflammatory activity, symptom burden, and laryngoscopic severity and to be an independent association with LPR after adjustment for major confounders. A pepsin-based combined biomarker-clinical model was able to discriminate better than pepsin alone. The pepsin gradient observed is biologically plausible as pepsin is of gastric origin and is a direct measure of the exposure to refluxate in the proximal portion of the gastrointestinal tract. The magnitude and diagnostic performance are comparable (but slightly superior to) the AUC of 0.767 reported by Liu *et al.* (2025) for fasting hypopharyngeal saliva. They had a very specific cutoff value of 29.62 ng/mL with limited sensitivity and highlighted the fact that they were only able to measure one sample of the fasting state. We used three-time samples which probably contributed to increased capture, especially after feeding and at night. Lechien *et al.* (2024b) also demonstrated that there are differences between the detection yield and accuracy of measurements taken during fasting and at bedtime ^[14]. The literature still is heterodox. Jing *et al.* (2023) concluded that salivary pepsin alone is not a good diagnostic tool and the accuracy is dependent on the cut-off and reference standard ^[7]. Li *et al.* (2024) also stressed the clinical utility variability ^[15]. The present study has two biases, group allocation was done according to HEMII-pH, not the pepsin test under study and non-LPR chronic laryngitis was used as disease control. The latter is important as specificity is overstated for reflux as

opposed to inflammation in general, when compared to healthy volunteers. The positive relationships of pepsin with IL-6, hs-CRP and TNF- α suggest (but do not establish) a connection between repeated mucosal exposure and the activation of the systemic inflammatory response. Pepsin may cause damage to the epithelial barrier of the larynx and trigger inflammation even at slightly acidic pH values. Liu *et al.*, 2021 and Cui *et al.*, 2024 reviewed the LPR mucosal biology, which includes cytokine recruitment, changes in epithelial permeability, and defects in protective responses ^[4,16]. In addition, recent mechanistic studies suggest that sterile inflammation of the laryngopharynx is also related to pepsin-dependent inflammasome activation ^[5]. IL-6 was the most powerful systemic correlator and the only one that was independent in the logistic model. This is in line with the idea that IL-6 acts upstream of the synthesis of hepatic CRP and that it is involved in the integration of the signals from the tissues and the acute-phase response system. However, when added together with IL-6, and hs-CRP, the latter two were independent and TNF- α was elevated but lost independence, possibly due to shared inflammatory variance. The anemone-like variation of non-LPR chronic laryngitis versus controls suggests that there is no unique systemic activation in reflux, and further supports the use of a multi-marker approach. The NLR, PLR and SII were increased in LPR associated disease and were an inexpensive adjunctive information. These ratios are an overview of changes in innate immune cells, adaptive immune cells and in platelets, but are influenced by smoking, obesity, infection, metabolic diseases and medications. They have lower AUCs and when statistically adjusted for independent SII failed to remain significant, suggesting that they should not be used in place of objective reflux testing or quantitative pepsin measurement. They could

also have a practical applications as readily available context markers, when cytokine assays are not available. Pepsin was correlated with RSS-12 and RSA-10 and showed an increase with the severity of the laryngoscopy. This further confirms the weak symptom/sign correlation found by Liu *et al.* (2025) and suggests a dose–response correlation. However, symptom scores reflect different aspects and are affected by non-reflux mechanisms^[17]. The high inter-rater reliability observed in the present study is due to the standardization of the recordings, the blinded dual review and the use of the concise RSA-10 which was developed with the aim of increasing the reliability of the recordings^[11]. Pepsin alone was good for discrimination at 48.5 ng/mL, but the optimum cut-off value depends on the type of assay and the protocol used and is not necessarily applicable to other laboratories. The addition of IL-6 and hs-CRP led to an increase in the AUC and the clinical covariates gave a bootstrap-corrected AUC of 0.944. This facilitates a multimodal phenotyping approach as opposed to a single test approach. But this apparent increase could be a model optimism and model thresholds should be externally validated, assessed, and evaluated in cohorts with natural prevalence of the disease^[18]. The results confirm the current consensus of using a combination of symptoms, examination and objective reflux assessment for diagnosing LPR^[3,19]. However, salivary pepsin may be best used as a supportive non-invasive biomarker, or as a triage test, in conjunction with other tests such as HEMII-pH, particularly if a single HEMII-pH test is negative. Strengths are that there are three clinically-informative groups, objective reflux classification without pepsin, repeated timed saliva collection, quantitative blinded duplicate assays, standardized blood collection, validated RSS-12 and RSA-10 tools, blinded dual laryngoscopic scoring, confounder

adjustment, false-discovery-rate control, and bootstrap internal validation. There are a number of caveats to take into account. First, because of its case-control design, it is not able to provide information on temporality or causality. Secondly the equal group sizes reflect not clinical prevalence, but are sample specific, and PPV and NPV are therefore also sample specific. Third, the cytokines and pepsin are variables with biological variations, and were evaluated for a short-term period. Fourthly, there may be some residual confounding due to unmeasured diet, sleep, stress, oral health and environmental exposures. Finally, the three-sample protocol and suggested cut-off needs to be independently analytically validated. Sixthly, the model of regression is limited in size and is still optimistic even after being adjusted by the bootstrap approach. Lastly, there may be a restriction in generalizability due to using only one region sample in the study. The combined model should be validated in prospective multicenter cohorts and assay specific reference intervals should be defined. Re-confirmation of biomarkers pre and post phenotype directed therapy may be testing responsiveness and temporal relationships. Research is also warranted to compare salivary pepsin with other newer digestive markers, to describe the different types of acid and nonacid reflux, and to see if biomarker-directed therapy results in better patient outcomes and decreases unnecessary acid suppression.

Conclusions

There was a significantly increased concentration of salivary pepsin in objective LPR associated chronic laryngitis and it was independently associated with systemic inflammatory activity, symptom burden and laryngoscopic severity. Both IL-6 and hs-CRP provided additional diagnostic information, with the CBC-derived indices providing less but readily available

additional information. Combination of pepsin-inflammation-clinical model demonstrated a good internal discrimination, but further external validation, calibration, and clinical-utility testing are needed prior to routine use.

Ethics approval and consent to participate:

Scientific and ethical approval and administrative permissions for the study were obtained, and all the participants signed informed consent form in accordance with the Declaration of Helsinki.

Availability of data: Not applicable.

De-identified data can be made available from the corresponding author on reasonable request and with ethics approval.

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Authors distribution

All authors helped in the study conception or design, clinical recruitment and assessment, acquisition, analysis or interpretation of data, critical revision of the manuscript, approval of the final version and accountability for the work. The roles will be allocated on the title page of the journal on an individual basis.

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