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## Biomarker–Phenotype Relationships in Clinically Suspected Laryngopharyngeal Reflux–Associated Dysphonia: A Multidomain Analysis of Salivary Pepsin, Salivary pH, Reflux Symptoms, Laryngeal Findings, and Voice Handicap

Muhammad Rizal<sup>1</sup>, Muhammad Amsyar Akil<sup>1</sup>, Khaeruiddin HA<sup>1</sup>, Abdul Salam<sup>2</sup>

<sup>1</sup>Department of Otorhinolaryngology–Head and Neck Surgery, Faculty of Medicine, Hasanuddin University, Makassar, South Sulawesi, Indonesia

<sup>2</sup>Department of Nutrition, Faculty of Public Health, Hasanuddin University, Makassar, South Sulawesi, Indonesia

**ABSTRACT:** Laryngopharyngeal reflux (LPR) can present with dysphonia, yet the extent to which salivary biomarkers correspond to symptom burden, laryngeal findings, and voice-related disability remains unclear. This analytical cross-sectional study examined relationships among salivary pepsin, salivary pH, Reflux Symptom Index (RSI), Reflux Finding Score (RFS), and Voice Handicap Index-30 (VHI-30) in 21 treatment-naïve adults with clinically suspected LPR-associated dysphonia, defined by  $RSI \geq 13$  and  $RFS \geq 7$ . Salivary pepsin was quantified using sandwich ELISA, and salivary pH was measured with a digital pH meter. Ten pairwise associations were analyzed using Spearman correlations with 5,000-resample bias-corrected and accelerated bootstrap 95% confidence intervals. Benjamini–Hochberg false-discovery-rate correction was applied. Salivary pepsin and salivary pH showed weak correlations with RSI, RFS, and VHI-30, with all bootstrap confidence intervals crossing zero. Nominal positive associations were observed between RSI and RFS ( $\rho=0.556$ ,  $p=0.009$ ), RSI and VHI-30 ( $\rho=0.477$ ,  $p=0.029$ ), and RFS and VHI-30 ( $\rho=0.457$ ,  $p=0.037$ ), although none remained statistically significant after false-discovery-rate correction (all  $q \geq 0.090$ ). Clinical phenotype measures therefore showed numerically stronger interrelationships than their associations with salivary biomarkers, although none remained statistically significant after multiplicity correction. Single-time salivary pepsin and pH measurements may therefore not reliably reflect clinical severity in suspected LPR-associated dysphonia.

## 1. INTRODUCTION

Laryngopharyngeal reflux (LPR) involves the direct or indirect effects of gastroduodenal refluxate on the upper aerodigestive tract and commonly presents with dysphonia, throat clearing, cough, globus sensation, and other chronic laryngeal symptoms [1]. Establishing whether reflux is responsible for these manifestations remains challenging because symptoms and laryngoscopic findings are heterogeneous and insufficiently specific when interpreted in isolation [2]. Clinical symptoms, laryngeal findings, reflux physiology, and voice-related disability therefore capture different aspects of the LPR phenotype and should not be considered interchangeable.

Salivary pepsin has attracted interest as a non-invasive marker of proximal gastroduodenal reflux. However, its diagnostic performance is inconsistent. Meta-analytic evidence has demonstrated modest overall accuracy, while subsequent reviews show substantial variation according to assay technique, diagnostic threshold, sampling protocol, and reference standard [3–5]. Sampling strategy is particularly relevant: repeated measurements improve detection compared with single-time testing, and diagnostic yield varies according to collection timing [6–8].

Salivary pH has also been investigated as a potential non-invasive marker, although its clinical interpretation remains uncertain. HEMII-pH-confirmed LPR has been associated with more alkaline salivary pH than that observed in asymptomatic controls, indicating that LPR does not necessarily translate into salivary acidification [9]. Voice abnormalities and treatment-related improvements have also been documented across acid, weakly acid, and alkaline LPR phenotypes [10].

Whether these biochemical and clinical measures covary within the same patient remains insufficiently characterized. Therefore, this study examined the multidomain relationships among salivary pepsin, salivary pH, reflux symptoms, laryngeal findings, and voice handicap in adults with clinically suspected LPR-associated dysphonia.

## 2. MATERIALS AND METHODS

### 2.1 Study Design and Setting

This analytical cross-sectional study investigated the associations between salivary biomarkers and clinical phenotypes in adults with clinically suspected laryngopharyngeal reflux (LPR)-associated dysphonia. Participants were recruited between January and July 2026 from the Otorhinolaryngology–Head and Neck Surgery outpatient clinics of Hasanuddin University Hospital, Dr. Wahidin Sudirohusodo Hospital, and Haji Hospital, Makassar, Indonesia. Clinical assessments were performed at the participating hospitals, while saliva processing, salivary pH measurement, and pepsin quantification were conducted at the Hasanuddin University Medical Research Center.

### 2.2 Participants

Adults aged 18–80 years presenting with dysphonia were consecutively screened at the participating otorhinolaryngology outpatient clinics. Participants were eligible when clinically suspected LPR was present, operationalized according to the prespecified study criteria as a Reflux Symptom Index (RSI) score  $\geq 13$  and/or a Reflux Finding Score (RFS)  $\geq 7$ . Participants were required to be treatment-naïve for anti-reflux therapy and to provide written informed consent. Patients with reflux-related clinical findings without dysphonia, first-trimester pregnancy, or a history of laryngeal or pharyngeal surgery were excluded. Eligible patients were enrolled consecutively during the study period until the prespecified sample size was reached. All 21 enrolled participants completed the clinical and laboratory assessments and had complete data for all variables included in the analysis.

### 2.3 Sample Size Calculation

The minimum sample size was calculated a priori for detecting a correlation using Fisher's  $z$  transformation, assuming a two-sided  $\alpha$  of 0.05, 80% statistical power, and an anticipated correlation coefficient of 0.60. Sample-size determination based on the anticipated correlation magnitude is an established approach for correlation studies [11,12]. An effect magnitude of approximately 0.60 was considered plausible based on previous research in patients with voice disorders and laryngopharyngeal reflux, which reported correlations of this magnitude between laryngeal findings and objective pharyngeal reflux measurements [13]. Under these assumptions, the minimum required sample size was 20 participants. Twenty-one participants were included in the final analysis.

### 2.4 Clinical Phenotype Assessment

Clinical phenotyping included the RSI, RFS, and 30-item Voice Handicap Index (VHI-30). Reflux-related symptoms were assessed using the RSI, a nine-item self-administered instrument developed to quantify the severity of symptoms associated with LPR [14]. An RSI score  $\geq 13$  was used as one component of the clinical eligibility criteria. For the multidomain analysis, the total RSI score was retained as a continuous variable.

Laryngeal findings were assessed by fiberoptic laryngoscopy and quantified using the RFS, a standardized scoring system for laryngeal findings associated with LPR [15]. An RFS  $\geq 7$  was required for study inclusion, while the total RFS was analyzed as a continuous variable.

Voice-related impairment was assessed using the VHI-30, which evaluates the functional, physical, and emotional consequences of voice disorders [16]. The instrument comprises 30 items scored from 0 to 4, producing a total score ranging from 0 to 120, with higher scores indicating greater perceived voice handicap. For descriptive purposes, VHI-30 scores were categorized as mild (0–30), moderate (31–60), or severe (61–120). The continuous VHI-30 total score was used for correlation analyses. For the multidomain analysis, salivary pepsin concentration and salivary pH represented the biomarker domain, whereas RSI, RFS, and VHI-30 represented the clinical phenotype domain.

## 2.5 Saliva Collection and Processing

A single saliva sample was collected from each participant in the morning during the scheduled outpatient visit. The exact clock time was not standardized beyond morning collection. Participants were instructed to fast for at least 2 hours before sampling and to avoid smoking, coffee, tea, spicy foods, medications, and antiseptic mouthwash before collection. Before sampling, participants rested in a seated position for approximately 5 minutes. Approximately 5 mL of unstimulated whole saliva was then collected by passive drooling into a sterile collection tube. Samples were immediately maintained at approximately 4°C and transported to the laboratory within 1–2 hours. In the laboratory, samples were centrifuged for 20 minutes at  $1,000 \times g$  at 2–8°C to remove cellular debris. The clear supernatant was transferred into sterile microtubes. Approximately 1 mL was reserved for immediate pH measurement before freezing, and the remaining supernatant was stored at –20°C until pepsin analysis.

## 2.6 Salivary pH Measurement

Before freezing, approximately 1 mL of each saliva sample was reserved for pH measurement. Salivary pH was measured using a digital pH meter by immersing the electrode directly into the saliva aliquot. Measurements were recorded to two decimal places and analyzed as continuous values.

## 2.7 Salivary Pepsin Quantification

Salivary pepsin concentration was quantified using a commercially available sandwich enzyme-linked immunosorbent assay (Human PP [Pepsin] ELISA Kit, Cat. No. E-EL-H2050; Elabscience Biotechnology Inc., Houston, TX, USA). According to the manufacturer, the assay has a detection range of 62.5–4000 pg/mL and a sensitivity of 37.5 pg/mL. All reagents and samples were brought to room temperature (18–25°C) before analysis. Saliva samples were diluted 1:100 by mixing 5 µL of saliva with 495 µL of the supplied sample diluent. A volume of 100 µL of each standard and diluted sample was added to antibody-coated microplate wells and incubated for 90 minutes. After washing, 100 µL of biotinylated detection antibody solution was added and incubated for 60 minutes at 37°C, followed by washing and incubation with 100 µL of enzyme conjugate for 30 minutes. After subsequent washing, 100 µL of tetramethylbenzidine substrate was added and incubated for 15 minutes in the dark. The reaction was terminated with 50 µL of stop solution, and optical density was measured at 450 nm using a microplate reader. Pepsin concentrations were calculated from the standard curve, corrected for the 100-fold dilution factor, converted from pg/mL to ng/mL, and expressed in ng/mL for statistical analysis. All diluted study samples yielded concentrations within the stated analytical range.

## 2.8 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Macintosh, Version 30.0 (IBM Corp., Armonk, NY, USA). The distribution of continuous variables was evaluated using the Shapiro–Wilk test. Normally distributed continuous variables were summarized as mean  $\pm$  standard deviation, whereas non-normally distributed variables were reported as median and interquartile range (Q1–Q3). Categorical variables were summarized as frequencies and percentages.

The primary multidomain analysis evaluated all 10 unique pairwise associations among salivary pepsin concentration, salivary pH, RSI, RFS, and VHI-30 using Spearman rank correlation coefficients ( $\rho$ ). Spearman correlation was selected as a common estimator across the correlation matrix because of the small sample size and the non-normal distribution of at least one clinical measure.

Because the sampling distribution of correlation coefficients may be unstable in small samples, uncertainty around each Spearman correlation coefficient was additionally quantified using nonparametric bootstrap resampling. Participants were resampled with replacement, with the Spearman coefficient recalculated in each of 5,000 bootstrap samples. Bias-corrected and accelerated (BCa) 95% confidence intervals were used because this approach accounts for both bias and skewness in the

bootstrap distribution. Bootstrap resampling was used to estimate confidence intervals; the reported two-sided *p* values were obtained from the original Spearman correlation analyses.

Because 10 pairwise correlations were evaluated within the same multidomain analysis, these tests were treated as one family for multiplicity control. The Benjamini–Hochberg false discovery rate procedure was selected instead of a family-wise error-rate procedure because the analysis was exploratory and involved correlated clinical and biomarker measures, while preservation of statistical power was considered important. The 10 unadjusted two-sided *p* values were ranked from smallest to largest and converted to Benjamini–Hochberg-adjusted *q* values. A raw *p* < 0.05 was regarded as nominally significant, whereas *q* < 0.05 was required for statistical significance after multiplicity adjustment. Because the original sample-size calculation targeted a single anticipated correlation rather than the complete 10-comparison matrix, the multidomain analysis was considered exploratory.

Given the small sample size, multivariable covariate-adjusted models were not fitted because of the risk of unstable estimates and overfitting. Accordingly, all reported correlation coefficients represent unadjusted associations.

## 3. RESULTS AND DISCUSSION

### 3.1 Participant Characteristics and Multidomain Measures

A total of 21 participants with clinically suspected LPR-associated dysphonia were included, with complete data for all analyzed variables. Although eligibility permitted fulfillment of either the RSI or RFS threshold, all enrolled participants met both thresholds in the observed study sample. The mean age was  $49.62 \pm 16.42$  years, and 12 participants (57.1%) were male. Most participants were recruited from Hasanuddin University Hospital (52.4%). The median RFS was 9.00 (8.00–12.00), whereas the mean RSI, salivary pH, salivary pepsin concentration, and VHI-30 were  $19.48 \pm 4.05$ ,  $7.23 \pm 0.74$ ,  $135.80 \pm 92.65$  ng/mL, and  $59.24 \pm 23.57$ , respectively. More than half of the participants had severe voice handicap (52.4%) (Table 1).

**Table 1.** Participant characteristics and multidomain measures

| Variable                           | Overall (n=21)     |
|------------------------------------|--------------------|
| <b>Participant characteristics</b> |                    |
| Age, years                         | $49.62 \pm 16.42$  |
| Sex, n (%)                         |                    |
| Male                               | 12 (57.1)          |
| Female                             | 9 (42.9)           |
| Recruitment site, n (%)            |                    |
| Hasanuddin University Hospital     | 11 (52.4)          |
| Dr. Wahidin Sudirohusodo Hospital  | 6 (28.6)           |
| Haji Hospital                      | 4 (19.0)           |
| <b>Multidomain measures</b>        |                    |
| Reflux Symptom Index (RSI)         | $19.48 \pm 4.05$   |
| Reflux Finding Score (RFS)         | 9.00 (8.00–12.00)  |
| Salivary pH                        | $7.23 \pm 0.74$    |
| Salivary pepsin, ng/mL             | $135.80 \pm 92.65$ |
| Voice Handicap Index (VHI-30)      | $59.24 \pm 23.57$  |
| Voice handicap severity, n (%)     |                    |
| Mild                               | 2 (9.5)            |

|          |           |
|----------|-----------|
| Moderate | 8 (38.1)  |
| Severe   | 11 (52.4) |

Notes: Values are presented as mean  $\pm$  SD, median (Q1–Q3), or n (%), according to variable type and distribution. RFS is presented as median (Q1–Q3); other continuous variables as mean  $\pm$  SD. Abbreviations: RSI, Reflux Symptom Index; RFS, Reflux Finding Score; VHI, Voice Handicap Index.

### 3.2 Multidomain Associations Between Salivary Biomarkers and Clinical Phenotypes

Across the biomarker-related comparisons, salivary pepsin and salivary pH showed weak correlations with reflux symptoms, laryngeal findings, and voice handicap, with all BCa 95% confidence intervals crossing zero (Table 2). In contrast, positive nominal associations were observed among the clinical phenotype measures: RSI with RFS ( $\rho=0.556$ ,  $p=0.009$ ), RSI with VHI ( $\rho=0.477$ ,  $p=0.029$ ), and RFS with VHI ( $\rho=0.457$ ,  $p=0.037$ ). The overall correlation pattern is visualized in the Spearman correlation matrix (Figure 1), while the corresponding effect estimates and BCa 95% confidence intervals are displayed in the forest plot (Figure 2). After Benjamini–Hochberg correction for 10 pairwise comparisons, none of these associations remained statistically significant (all  $q \geq 0.090$ ).

**Table 2.** Pairwise associations across biomarker and clinical phenotype domains

| Domain pair                                  | Spearman $\rho$ | BCa 95% CI      | p value | BH-FDR q value |
|----------------------------------------------|-----------------|-----------------|---------|----------------|
| <b>Biomarker–biomarker</b>                   |                 |                 |         |                |
| Salivary pepsin – salivary pH                | –0.073          | –0.563 to 0.457 | 0.752   | 0.930          |
| <b>Biomarker–clinical phenotype</b>          |                 |                 |         |                |
| Salivary pepsin – RSI                        | –0.020          | –0.490 to 0.458 | 0.930   | 0.930          |
| Salivary pepsin – RFS                        | –0.097          | –0.518 to 0.347 | 0.676   | 0.930          |
| Salivary pepsin – VHI                        | 0.146           | –0.335 to 0.620 | 0.527   | 0.912          |
| Salivary pH – RSI                            | –0.139          | –0.567 to 0.304 | 0.547   | 0.912          |
| Salivary pH – RFS                            | –0.038          | –0.542 to 0.420 | 0.869   | 0.930          |
| Salivary pH – VHI                            | –0.313          | –0.673 to 0.143 | 0.168   | 0.420          |
| <b>Clinical phenotype–clinical phenotype</b> |                 |                 |         |                |
| RSI – RFS                                    | 0.556           | 0.109 to 0.850  | 0.009*  | 0.090          |
| RSI – VHI                                    | 0.477           | 0.054 to 0.810  | 0.029*  | 0.123          |
| RFS – VHI                                    | 0.457           | 0.155 to 0.662  | 0.037*  | 0.123          |

Notes:  $\rho$ , Spearman rank correlation coefficient; BCa, bias-corrected and accelerated. BCa 95% confidence intervals were estimated using 5,000 bootstrap resamples. Raw  $p$  values are two-sided. Benjamini–Hochberg false discovery rate (BH-FDR) correction was applied across all 10 pairwise comparisons. \*Nominal  $p < 0.05$ . No association remained statistically significant after multiplicity adjustment at  $q < 0.05$ . Abbreviations: RSI, Reflux Symptom Index; RFS, Reflux Finding Score; VHI, Voice Handicap Index.

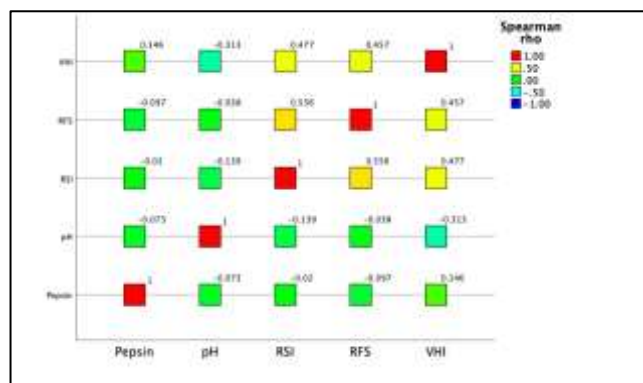


**Figure 1. Spearman correlation matrix across biomarker and clinical phenotype domains.** Cell values represent Spearman rank correlation coefficients ( $\rho$ ). RSI, Reflux Symptom Index; RFS, Reflux Finding Score; VHI, Voice Handicap Index.

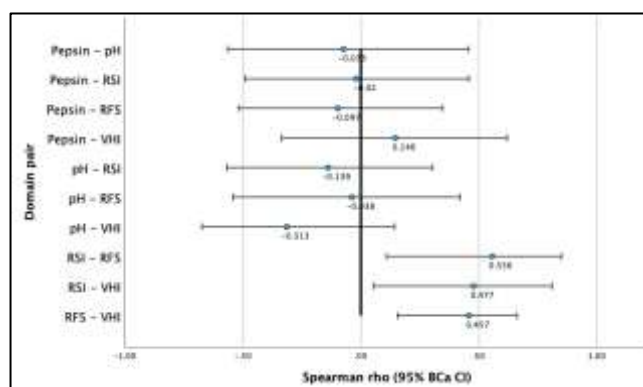


**Figure 2. Forest plot of pairwise correlations across biomarker and clinical phenotype domains.** Points represent Spearman rank correlation coefficients ( $\rho$ ), and horizontal lines indicate 95% bias-corrected and accelerated (BCa) bootstrap confidence intervals based on 5,000 resamples. The vertical reference line indicates  $\rho=0$ . RSI, Reflux Symptom Index; RFS, Reflux Finding Score; VHI, Voice Handicap Index.

### 3.3 Discussion

The principal finding of this study was a pattern consistent with relative biomarker–phenotype discordance. Salivary pepsin and salivary pH showed weak cross-sectional relationships with reflux symptoms, laryngeal findings, and voice handicap, whereas the clinical phenotype measures showed numerically stronger positive associations with one another. These latter associations were nominal and did not remain statistically significant after false-discovery-rate correction; therefore, they should be interpreted as an observed pattern rather than evidence of a unified clinical construct. This distinction is consistent with contemporary LPR frameworks, which emphasize that chronic laryngeal symptoms, laryngoscopic findings, reflux physiology, and biochemical markers represent related but non-equivalent dimensions of disease [1,2].

The weak pepsin–phenotype relationships are plausible in light of the substantial methodological and biological variability of salivary pepsin testing. Jing et al. [3] reported pooled sensitivity of 61%, specificity of 67%, and an area under the curve of 0.67, while Hiernaux et al. [4] documented wide ranges of sensitivity and specificity across both Peptest and ELISA platforms. A more recent Peptest-specific meta-analysis found that diagnostic performance varied according to the selected threshold and that multiple-time sampling improved sensitivity compared with single-time sampling [5]. Prospective studies similarly indicate that diagnostic yield varies with both sampling frequency and collection timing [6–8]. Importantly, findings across studies are not uniform. Klimara et al. [17] identified associations between waking salivary pepsin and selected MII-pH parameters and RSI, whereas Lechien et al. [18] found no significant relationship between hypopharyngeal reflux events and salivary pepsin concentration. A single morning measurement may therefore capture only a limited temporal window of reflux exposure rather than the cumulative burden relevant to persistent symptoms or established tissue injury.

Biological plausibility for pepsin-mediated laryngeal injury nevertheless remains strong. Tan et al. [19] demonstrated that pepsin increased reactive oxygen species and activated the TXNIP/NLRP3/caspase-1/IL-1 $\beta$  inflammatory pathway in laryngeal tissue and epithelial-cell experiments. In an in-vitro rat vocal-fold model, acidic pepsin disrupted epithelial tight-junction integrity and increased intercellular permeability more strongly than acid alone [20]. A systematic review of human laryngeal tissue further identified inflammatory-cell infiltration, cell-junction proteolysis, intercellular-space dilatation, oxidative stress, DNA damage, and impairment of mucosal defense mechanisms in LPR-related specimens [21]. Collectively, these findings support a mechanistic link between refluxate exposure and laryngeal tissue injury, while also explaining why salivary pepsin measured at a single time point may not closely parallel established mucosal changes or perceived voice disability. Refluxate composition is itself heterogeneous and may include acid, pepsin, bile acids, trypsin, and other gastroduodenal components [22].

The findings involving salivary pH require similar caution. Recent evidence challenges the intuitive assumption that LPR should consistently produce more acidic saliva. In patients with HEMII-pH-confirmed LPR, Lechien et al. [9] reported a more alkaline mean salivary pH than in asymptomatic controls and identified an association between salivary pH and reflux symptom burden. Chethana et al. [23] likewise observed significantly higher salivary pH in clinically defined LPR than in controls. The absence of comparable pH-RSI or pH-RFS relationships in the present study therefore differs from these reports and may reflect the small sample, single-time sampling, differences in case definition, and the fact that salivary pH is not equivalent to the pH of individual hypopharyngeal reflux events. This distinction is clinically relevant because acid, weakly acid, and alkaline LPR phenotypes can all be associated with voice impairment and treatment-related voice changes [10].

The nominal positive relationships among RSI, RFS, and VHI-30 suggest partial coherence within the clinical phenotype, although previous evidence indicates that such coherence is not uniform. In patients with voice complaints, Włodarczyk et al. [13] found that a laryngoscopic severity scale correlated strongly with RFS, more modestly with RSI, and less consistently with VHI. Conversely, HEMII-pH-confirmed cohorts have shown parallel post-treatment improvements in reflux symptom and sign scores, VHI, perceptual dysphonia, and several acoustic and aerodynamic voice measures [10]. A recent systematic review similarly found that VHI and several perceptual and acoustic measures generally improve during LPR treatment, although objective confirmation of LPR and comprehensive multidimensional voice assessment were uncommon across studies [24]. Thus, symptom burden, visible laryngeal findings, and perceived voice disability may overlap while still representing distinct clinical constructs.

Clinically, the present findings do not support using a single salivary pepsin or salivary pH measurement as a surrogate for the severity of LPR-associated dysphonia. Current international consensus and European guidance favor multidimensional assessment and objective reflux monitoring, particularly HEMII-pH, when confirmation of LPR is required [1,25]. Strengths of this study include simultaneous assessment of biochemical, symptom, laryngoscopic, and patient-reported voice domains, together with bootstrap confidence intervals and multiplicity correction. Interpretation remains limited by the small cross-sectional sample, single-time saliva collection, lack of HEMII-pH confirmation, absence of acoustic, aerodynamic, or stroboscopic voice measures, and lack of multivariable adjustment for potential confounders. Because only 21 participants were available, covariate-adjusted models were not fitted to avoid overfitting; therefore, the reported effect estimates should be interpreted as unadjusted exploratory associations, and residual confounding cannot be excluded.

## 4. CONCLUSION

Among adults with clinically suspected LPR-associated dysphonia, salivary pepsin and salivary pH showed limited cross-sectional correspondence with reflux symptoms, laryngeal findings, and voice handicap, whereas the clinical phenotype measures showed numerically stronger correlations with one another. These exploratory findings support a multidimensional approach and suggest that single-time salivary biomarkers should not be assumed to reflect clinical severity. Prospective studies incorporating repeated biomarker sampling and objective reflux monitoring are warranted.

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## AUTHOR CONTRIBUTIONS

**Muhammad Rizal:** Conceptualization, methodology, investigation, data collection, data curation, formal analysis, project administration, visualization, writing—original draft, writing—review and editing.

**Muhammad Amsyar Akil:** Conceptualization, methodology, supervision, validation, interpretation of findings, writing—review and editing.

**Khaerudin HA:** Conceptualization, clinical methodology, supervision, validation, interpretation of findings, writing—review and editing.

**Abdul Salam:** Methodology, formal analysis, validation, interpretation of findings, writing—review and editing.

All authors approved the final manuscript and agreed to be accountable for their respective contributions.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## FUNDING

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## ETHICS STATEMENT

The study was approved by the Research Ethics Committee of the Faculty of Medicine, Hasanuddin University (approval No. 548/UN4.6.4.5.31/PP36/2026; protocol No. UH26030371). Written informed consent was obtained from all participants before enrollment. Participant information and biological specimens were coded to maintain confidentiality. The study was conducted in accordance with the principles of the Declaration of Helsinki.

## DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request, subject to applicable ethical and institutional restrictions.

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