

Human Vocal Fold Tissue Modifications Related to Laryngopharyngeal Reflux Disease: A Systematic Review[☆]

*Guangjin Chen, and *†‡§ Jérôme R. Lechien, *Mons, ‡Brussels, Belgium, †Paris, and §Poitiers, France

Summary: Background. The vocal fold tissue modifications and related dysphonia caused by laryngopharyngeal reflux disease (LPRD) remain a controversial topic in laryngology. Investigation of human vocal fold tissue exposed to reflux content can provide valuable insights. This systematic review aimed to summarize the current knowledge about LPRD-induced human vocal fold tissue modifications to better understand LPRD pathophysiology and LPRD-related voice disorders.

Methods. A PubMed, Embase, and Web of Science database search was carried out by two investigators for studies investigating human laryngeal mucosa injuries and histological modifications related to LPRD refluxate, and their potential mechanistic associations with voice quality impairments according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses statements.

Results. Of 389 retrieved articles, 24 experimental studies met the inclusion criteria. Studies demonstrate that laryngeal, particularly vocal fold, biopsies of patients with suspected LPRD reveal a substantial number of histological and functional alterations, including inflammatory cell infiltration, cell junction proteolysis, intercellular space dilatation, pepsin-induced cell DNA damage, and increases in oxidative stress mediators and tissue injuries. Functional impairment of defensive mechanisms through downregulation of carbonic anhydrases (CA III) and protective mucins (MUC2, MUC4, and MUC5AC) can consist of favoring factor of tissue injuries. Emerging studies reported evidence of tissue remodeling through matrix metalloproteinase activation and metabolic alterations included increased Glut-1 and sphingosine pathway activation, potentially linking LPRD to leukoplakia development. No studies addressed the potential effects of elastase, bile salts, trypsin, and lipases in non-acidic (weakly acidic or alkaline) gaseous environment.

Conclusion. This systematic review demonstrates that LPRD and pepsin induce cellular alterations in vocal fold and laryngeal tissues, highlighting potential pathogenic mechanisms and identifying biomarkers that may guide future diagnostic and therapeutic strategies.

Key Words: Laryngeal—Vocal fold—Mucosa—Epithelium—Modification—Injury—Voice—Gastroesophageal—Laryngopharyngeal—Reflux.

INTRODUCTION

Laryngopharyngeal reflux disease (LPRD) is a disease of the upper aerodigestive tract resulting from the direct and/or indirect effects of gastroduodenal content reflux, inducing morphological and/or neurological changes in the upper aerodigestive tract.¹ It has long been suggested that morphological changes can affect the vocal folds,² and are, consequently, associated with the development of LPRD-related dysphonia,^{2,3} while also promoting the development of benign vocal fold lesions.⁴ However, still today, the causal relationship between LPRD and dysphonia remains controversial,^{5,6} as objective documentation of macroscopic reflux-induced vocal

fold lesions remains difficult in clinical practice. This issue was addressed in an increasing number of studies investigating the vocal fold morphology, histology, and molecular functioning when exposed to gastroduodenal content.

This systematic review aimed to summarize the current knowledge about LPRD-induced human vocal fold tissue modifications to better understand LPRD pathophysiology and LPRD-related voice disorders.

MATERIALS AND METHODS

This review was conducted by two independent investigators (G.C. and J.R.L.) with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses checklist.⁷ The criteria for considering studies were based on population, intervention, comparison, outcome, timing, and setting framework.⁸

Types of Studies: The literature search included prospective, retrospective, or cross-sectional studies published between January 2000 and March 2025 in English-language peer-reviewed journals investigating histological or physiological changes of vocal fold/laryngeal mucosa exposed to LPRD contents in human specimens. Clinical studies, animal model studies, and nonexperimental studies, such as case reports, letter, and comments, were excluded.

Population: The criteria used for the LPRD diagnosis were extracted. The diagnosis of LPRD was considered as

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From the *Department of Surgery, UMONS Research Institute for Health Sciences and Technology, University of Mons (UMons), Mons, Belgium; †Department of Otolaryngology and Head and Neck Surgery, Foch Hospital, School of Medicine, UFR Simone Veil, Université Versailles Saint-Quentin-en-Yvelines (Paris Saclay University), Paris, France; ‡Department of Otolaryngology and Head and Neck Surgery, CHU Saint-Pierre, Brussels, Belgium; and the §Department of Otolaryngology, Elsan Hospital of Poitiers, Poitiers, France.

Address correspondence and reprint requests to: Jérôme R. Lechien, Department of Surgery, UMONS Research Institute for Health Sciences and Technology, University of Mons (UMons), Mons, Belgium. E-mail:

Jerome.Lechien@umons.ac.be

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confirmed for patients with more than one hypopharyngeal reflux event at the 24-hour hypopharyngeal-esophageal multichannel intraluminal impedance-pH monitoring (HEMII-pH).¹ The use of oropharyngeal pH monitoring, dual-, or triple-probe pH monitoring with pharyngeal pH sensor can support but not confirm the diagnosis.¹ Patients selected through the use of validated patient-reported outcome questionnaires [eg, reflux symptom index (RSI),⁹ reflux symptom score (RSS)¹⁰] and validated sign instruments [eg, reflux finding score (RFS),¹¹ reflux sign assessment¹²] were considered as individuals with a suspected LPRD. Patients with LPRD symptoms completing gastroesophageal reflux disease (GERD) diagnosis criteria (eg, Montreal, Lyon consensus)¹ were suspected of LPRD diagnosis.

Outcomes: Data from *in vitro* human-derived cell line experiments and *in vivo* laryngeal biopsy specimen studies were extracted by the two independent investigators. The primary outcomes were data describing potential association or mechanistic relationship between reflux content and mucosal morphology, histology, and functioning changes (*in vivo* studies). The features related to the reflux content may include pH variations and gastroduodenal enzymatic activity (specifically pepsin, bile salts, elastase, trypsin, and lipases). The comprehensive assessment of tissue response consisted of cellular morphology and ultrastructural modifications, epithelial integrity disruption, intercellular junction alterations, pro-inflammatory cytokine expression patterns, inflammatory infiltrate characterization, extra-, intra-, and transmembrane protein expression impairments, and all alterations of mucosal defense mechanisms. Due to the methodological differences between *in vivo* and *in vitro* study findings, *in vitro* investigations were discussed apart of the *in vivo* results.

Intervention and Comparison: Intervention consisting of application to reflux/gastroduodenal content into human vocal fold cell/tissue was considered. Evolution of mucosal morphology, histology, and functioning from pretreatment to post treatment of LPRD was similarly considered as an intervention.

Time and Setting: There were no strict criteria for time and setting.

Search strategy

The two investigators independently conducted the PubMed, Embase, and Web of Science databases for relevant peer-reviewed publications related to human vocal fold/laryngeal tissue modifications in LPRD. The following keywords were used for the search strategy: Larynx; Laryngeal; Reflux; Laryngopharyngeal; Gastroesophageal; Tissue; Human; Vocal Fold; Cord; and Outcomes. The studies reporting database abstracts, available full texts, or titles with the search terms were considered. The research findings have been reviewed for relevance and the reference lists of state-of-the-art or systematic reviews were examined for additional references. The included studies were analyzed for the number of patients/specimens, study design,

inclusion and exclusion criteria, demographics, and outcomes.

RESULTS

The systematic literature search yielded 1393 publications across three electronic databases: 570 from PubMed, 398 from Web of Science, and 425 from Embase (Figure 1). After removing 575 duplicates, 818 articles remained for initial screening. Title and abstract evaluation excluded 429 publications that failed to meet inclusion criteria. Subsequent full-text assessment of the remaining 389 articles identified 44 eligible studies examining the association between LPRD and vocal fold tissue pathology: 19 experimental animal studies and 25 human tissue specimen analyses (Table 1).^{13–36} One human study was excluded from further analysis as it examined only systemic biomarkers in peripheral blood samples of patients with suspected LPRD, rather than investigating laryngeal tissue pathology directly. The analysis of animal studies was provided in another systematic review discussing animal models in LPRD.³⁷

There were 4^{13,17,18,21} uncontrolled and 20 controlled studies.^{14–16,19,20,22–34} The *in vivo*/biopsy specimen studies primarily analyzed histopathological, functional, or gene expression changes in laryngeal tissues exposed to pepsin or gastroduodenal contents (Table 1). Five studies were conducted on cell cultures (*in vitro*),^{17,18,30,32,33} and their results were described in Appendix 1.

The list of the markers investigated in studies and their related roles in human physiology are available in Table 2. The comprehensive patterns of reflux-mediated pathogenesis in mucosal damage progression and functional impairment are summarized in Table 3.

Cell junctions and intercellular spaces

Cell junctions and intercellular spaces were studied in epithelial cells from vocal folds,^{14,16,21} ventricles,^{14,16} and posterior commissure.^{16,21,24} Filho et al reported epithelial cell findings from vocal folds with leukoplakia, which differs from other studies that focused on laryngeal tissue without benign lesions.³⁶

Histopathological analyses revealed alterations in patients with laryngopharyngeal reflux symptoms and findings, including dysregulated expression of cell junction proteins (eg, E-cadherin), intercellular dilatations, and inflammatory cell infiltration in laryngeal epithelial specimens (Table 1).

The expression of E-cadherin was reduced in vocal fold, posterior commissure, and ventricle tissues of suspected LPRD patients compared with controls in three studies,^{14,16,21} while β -catenin expression appeared unchanged.²¹ Samuels et al reported in an *in vitro* model that the proteolytic cleavage of E-cadherin was related to the activity of pepsin in vocal fold cells.³³ Only Filho et al did not find lower E-cadherin expression in LPRD tissues compared to others (leukoplakia), whereas regardless of

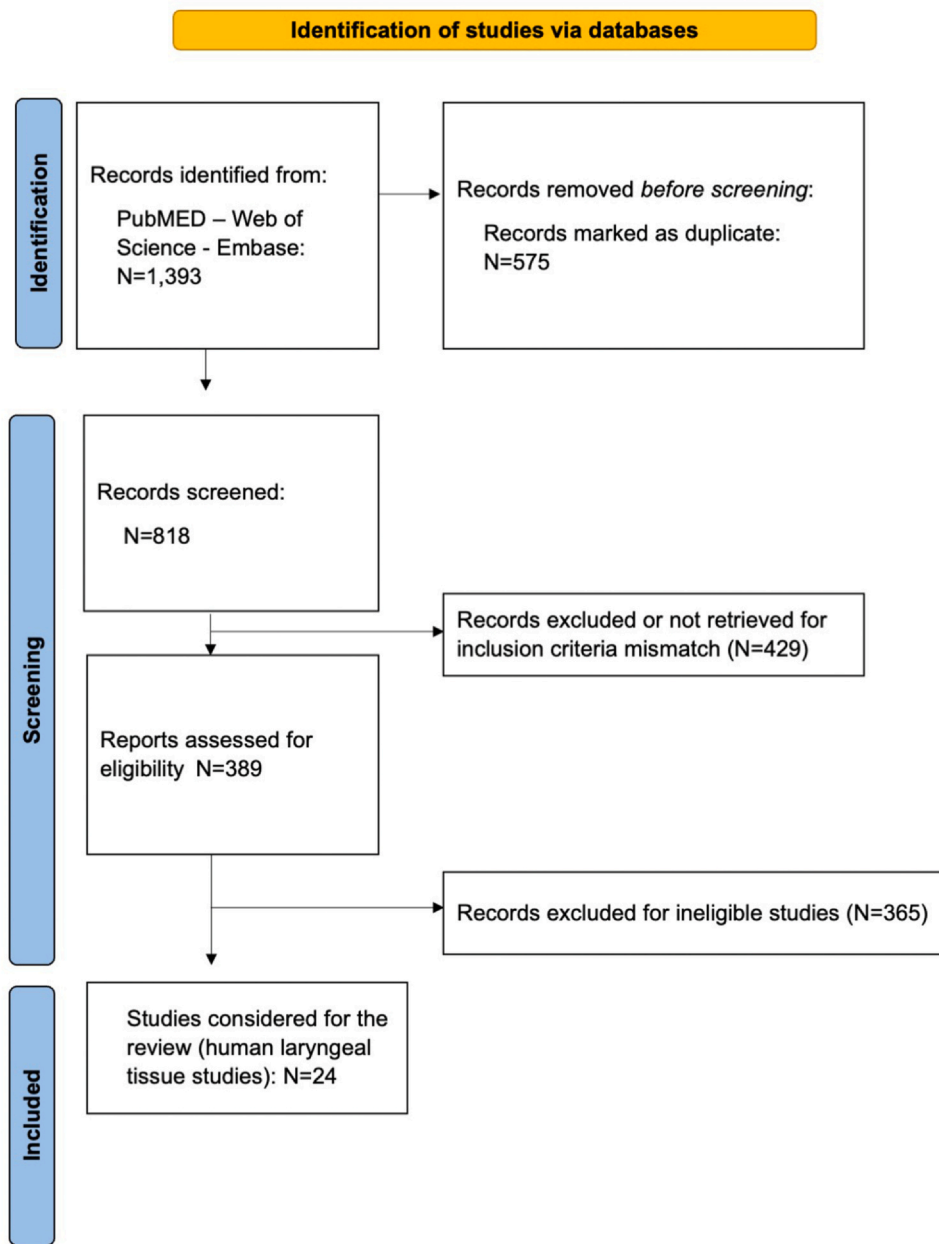


FIGURE 1. Flowchart.

the type of histological lesion, the authors demonstrated that patients with signs of laryngopharyngeal reflux had a lower expression of cathepsin B.³⁶ Vaezi et al did not observe significant differences in intercellular space, basal cell hyperplasia, intraepithelial lymphocytosis, and infiltrate between posterior commissure cells of suspected LPRD, GERD, and asymptomatic patients.²⁴ Cornulin is an epithelial differentiation marker involved in mucosal barrier integrity and response to stress. This marker was over-expressed in posterior commissure and arytenoid tissues of patients with severe LPRD symptoms (RSI) and findings (RFS).²⁹ These findings suggest that gastroduodenal content exposure may compromise epithelial cell integrity in the laryngopharyngeal region.²⁹

Inflammatory cell infiltration

Inflammation, mucosa injuries, or DNA damages were attributed to extra- and intracellular pepsin in four studies.^{16,18,25,31} A myriad of immune cells (eg, CD8 lymphocytes, MHC β 2m, MHC CD1d, and Treg) were found in the luminal and basal layers of vocal folds.^{20,23} By contrast, B cells, CD3 γ and CD3 δ T cells, neutrophils, eosinophils, monocytes, and MHC class I- and MHC class II-expressing cells appeared not to be involved in the inflammatory process of LPRD-exposed tissues.^{20,23,28} Inflammatory processes were indirectly assessed by Ylitalo et al through analysis of fibroblast mRNA expression in tissue biopsies from laryngeal ventricles and posterior commissure.¹⁷ As reported in Table 1 and 2, a myriad of

TABLE 1.
Features of Studies

References	Design	LPRD Diag.	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Axford et al 2001 ¹³	Prospective	N.P.	Vocal folds (a), post. com. (b)	IHC and WB	Expression: CA I (a, b), CA II (a, b)	+/-
Johnston et al 2003 ¹⁴	Uncontrolled Prospective	N.P.	n = 9 sLPRD Vocal folds (a), ventricle (c)	IHC and WB	CA III (a, b) Gene expression of CA I-II/CA III	-/+ Gr1 = Gr2/Gr2 > Gr1
	Controlled		post. com (b)		CA III expression — sympt. Correlation	S
Johnston et al 2004 ¹⁵	Prospective	Dual-probe	Gr1: 26 sLPRD Gr2: 19 CT Vocal folds (a), ventricles (b)	IHC and WB	E-cadherin expression (a,b,c) MUC4 and 5AC expression Pepsin tissue level	20/51 (37%) Gr2 > Gr1 Gr1 > Gr2 (a, b)
	Controlled	pH metry	Gr1: 9 sLPRD, Gr2: 12 CT		Pepsin-CA III depletion correlation	+ (a, b)
Gill et al 2005 ¹⁶	Prospective	RSI > 11 and	Vocal folds (a), ventricles (c)	IHC and WB	Intracellular pepsin	Gr1 > Gr2 (a, b, and c)
	Controlled	RFS > 5 or pH metry	post. com (b) Gr1: 18 sLPRD Gr2: 12 CT		E-cadherin CA III Pepsin and lack of CA III correlation	Gr2 > Gr1 (a, b, and c) Gr2 > Gr1 (a, c) S (a)
Franchi et al 2007 ¹⁹	Prospective	pH metry	post. com	Microscopy	Intercellular spaces dilatation	Gr1 > Gr2
Rees et al 2008 ²⁰	Controlled Prospective	(GERD) RSI > 21	Gr1: 15 sLPRD-Gr2: 7 CT Posterior vocal fold tissue	IHC	B cells, neutrophils, and eosinophils,	Gr1 = Gr2
	Controlled		Gr1: 12 sLPRD	Immune cell	CD8 lymphocytes (luminal and basal layers)	Gr1 > Gr2
			Gr2: 11 CT	Infiltrate	Monocytes, expression of MHC I, II	Gr1 = Gr2
Reichel et al 2008 ²¹	Prospective	Dual-probe	Vocal folds, post. com.	IHC	β 2 microglobulin (deepest layers)	Gr1 > Gr2
	Uncontrolled	pH metry (some patients)	Gr1:14 sLPRD (pH metry) Gr2: 7 sLPRD (symptoms)		E-cadherin	Gr2 > Gr1
Samuels and Johnston 2008 ²²	Prospective	RSI and RFS	Posterior commissure	RT-PCR	β -Catenin	Gr1 = Gr2
	Controlled Prospective	RSI 21-30	Gr1: 3 sLPRD, Gr2: 2 CT Human laryngeal samples	IHC	Mucin Gene Expression	Gr2 > Gr1 Gr1 > Gr2
Vaezi et al 2010 ²⁴	Controlled Prospective	N.P.	Gr1: 12 sLPRD-Gr2: 11 CT Posterior commissure	Microscopy	MUC2, 3, 5AC, and 5B CD161, MHC β 2m, and MHC CD1d MHC I, II	Gr1 = Gr2 Gr1 = Gr2 = Gr3

TABLE 1 (Continued)

References	Design	LPRD Diag.	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Jiang et al 2011 ²⁵	Controlled		Gr1: 18 sLPRD, Gr2: 20 GERD Gr3: 15 CT		Eosinophil, polymorphonuclear intercellular spaces, basal cell hyperplasia <i>Intracellular Pepsin</i>	Gr1 = Gr2 = Gr3 Gr1 = Gr2 = Gr3
	Prospective Controlled	Dual-probe pH metry	Posterior commissure Gr1: 7 acid, Gr1: 8 nonacid LPRD Gr2: CT	IHC		Gr1 > Gr2 Gr1a = Gr1na
El-Sayed et al 2014 ²⁶	Prospective	N.P.	Ventricles (a), vocal folds (c)	In situ	MUC1, 3, and 4	CT = sLPRD (a, b, c)
	Controlled		Posterior commissure (b): 27 sLPRD Healthy laryngeal biopsies: 3 CT	hybridization	MUC2 MUC5AC	CT > sLPRD (b), CT = sLPRD (a, c) CT > sLPRD (a, b), CT = sLPRD (c)
Min et al 2016 ²⁷	Prospective Controlled	RSI > 13 RFS > 7	Posterior commissure Gr1 = 10 sLPRD, Gr2 = 18 non-LPRD	IHC	<i>Gene Expression (CT)</i> MUC1, 3, 4/2, and 5AC <i>Expression</i> CA III, Hsp70	+ (a, b, c)/+ (a, c) Gr1 > Gr2
Jetté et al 2017 ²⁸	Prospective Controlled	MII-pH	False vocal fold exposed to Gr1: tobacco (n = 2) Gr2: refluxate and tobacco (n = 7) Gr3: refluxate (n = 21), Gr4: none (n = 12)	qPCR	<i>Gene Expression</i> T cells (CD3 γ CD3 δ /GAPDH) Treg cells (TSDR/GAPDH)	<i>Smoke/Reflux</i> Negative relation/NS No relation/no relation
Wood et al 2018 ²⁹	Prospective	RSI > 12	False vocal fold (a), vocal folds (b),	qRT-PCR	ImmunoCRIT value (Treg/T)	Positive relation/NS
	Controlled	RFS > 6	arytenoid (c), post. com. (d) Gr1: 10 sLPRD-Gr2: 9 RSI < 12		<i>False Vocal Folds and Vocal Folds</i> IL-6, -8, PTGS2, CRNN, CD1d, and VEGFA TGF β -1, MGMT, MUC1, 2, 3B, 4, 5B, 6, and 7 CA III, CDH1 KRT14, CRNN, and CDH1 CD1d, TGF β -1, MUC2, and MUC5B KRT14 MUC5B Pepsin in biopsy, DNA damage	NS NS NS Gr1 > Gr2 (c) Gr2 > Gr1 (c) Gr1 > Gr2 (d) Gr2 > Gr1 (d) Gr1 > Gr2, Gr1-2 > Gr3
Dai et al 2020 ³¹	Prospective	N.P.	Vocal fold polyp biopsies/ saliva	IHC		
	Controlled		Biopsies and saliva Gr1: 22 pepsin+, Gr2: 8 pepsin-	ELISA	8-OHdG, p-H2AX Pepsin in saliva	Gr1 > Gr2 Gr1 > Gr2

TABLE 1 (Continued)

References	Design	LPRD Diag.	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Chen et al 2024 ³⁵	Prospective Controlled	RSI > 13 RFS > 7	Vocal fold leukoplakia biopsy Gr1: 6 sLPRD Gr2: 9 non-LPRD	IHC qPCR WB	Sphk1, S1P, and ki-67 Sphk1, S1P, S1PR1, IL-6, and TNF α Proliferation-related protein p-AKT, p-ERK Cathepsin B E-cadherin	Gr2 > Gr1 Gr1 > Gr2 Gr1 > Gr2 Gr2 > Gr1 Gr1 = Gr2
Filho et al 2024 ³⁶	Prospective Controlled	Clinical diagnosis	Vocal fold leukoplakia biopsy Gr1: 21 sLPRD, Gr2: 11 non-LPR	IHC		
Tan et al 2024 ³⁴	Prospective Controlled	N.P.	Human laryngeal biopsy Gr1: 40 pepsin-positive Gr2: 8 pepsin-negative	DHE Kit IHC	ROS Caspase-1, IL-1 β and IL-18	Gr1 > Gr2 Gr1 > Gr2 Gr1 = Gr2

Abbreviations: AGJ+P, artificial gastric juice + pepsin; CA, carbonic anhydrase; CT, controls; Diag, diagnosis; GERD, gastroesophageal reflux disease; IHC, immunohistochemistry; (s)LPRD, (suspected) laryngopharyngeal reflux disease; MUC, mucin; NP, not provided; NS, nonsignificant; post.com, posterior commissure; RFS, reflux finding score; RSI, reflux symptom index; RT-PCR, reverse transcription-polymerase chain reaction; S, significant; WB, Western blot.

cytokines and chemokines were investigated in laryngeal biopsies, with expression patterns varying according to both the pH environment and the duration of pepsin exposure to tissues.¹⁷ Tan et al similarly identified several inflammatory mediators, including caspase-1 and IL-1 β , that correlate with pepsin detection in laryngeal tissues. Their investigation further demonstrated increased oxidative stress as a component of the inflammatory cascade.³⁴

This review reports that some biomarkers of DNA damage and oxidative stress (8-hydroxy-2'-deoxyguanosine, phosphorylated H2A histone family member X, and reactive oxygen species) have been linked to pepsin-induced epithelial damages in vocal fold polyps³¹ and laryngeal tissues.³⁴ Various matrix metalloproteinases (MMPs), enzymes responsible for degrading extracellular matrix components during tissue remodeling processes, were detected at differential expression levels in vocal fold epithelial cells exposed to pepsin.^{30,33} These findings support the hypothesis that LPRD-associated pepsin exposure may contribute to laryngeal tissue destruction and subsequent pathological remodeling (Tables 1 and 2).^{30,33}

Chen et al examined the potential contribution of LPRD to leukoplakia development by investigating sphingosine-1-phosphate (S1P) and sphingosine kinase 1 (SphK1), mediators involved in cellular proliferation, survival, and immune regulation.³⁵ Their analysis revealed that patients presenting with both suspected LPRD (defined by RSI > 13 and RFS > 7) and leukoplakia demonstrated significantly elevated expression of S1P, SphK1, IL-6, and TNF- α compared with subjects with leukoplakia without LPRD features. These findings suggest that LPRD may promote a proliferative microenvironment conducive to leukoplakia development.³⁵

The potential association between LPRD and leukoplakia was further studied by Ao et al, who demonstrated that upregulated glucose transporter-1 (Glut-1) expression may contribute to vocal fold leukoplakia pathogenesis through enhanced expression of laryngeal H⁺/K⁺-ATPase. This proton pump augmentation subsequently facilitates reactivation of absorbed pepsin, leading to laryngeal mucosal injury (apoptosis), and related wound healing processes (cell migration and proliferation).³²

Functional cellular changes

Carbonic anhydrases (CAs) and mucins (MUCs) were the most commonly studied molecules involved in laryngeal tissue function and defense mechanisms against LPRD (Tables 1 and 3).

CAs are involved in the defense of mucosa against acidity/gastroduodenal content aggression. Axford et al reported that the expression of CA III was increased in esophagitis as compared with normal esophageal tissue, while vocal folds and posterior commissure hypertrophy expressed CA types I and II, demonstrating potential defense mechanisms against refluxate aggression.¹³ Johnston et al showed a significant depletion of CA III in vocal fold mucosa of suspected LPRD compared with controls; the

TABLE 2.
Molecules and Outcomes Evaluated in Laryngeal Tissues

Molecules and Outcomes	Abbreviation	Definition/Role
Activating transcription factor 3	ATF-3	Transcription factor involved in cellular stress response and inflammation regulation
Protein kinase B (phosphorylated)	p-AKT	Key signaling protein that regulates cell survival, growth, proliferation, and metabolism
Beta-2 microglobulin	β 2m	Component of MHC class I molecules, essential for antigen presentation and stability of MHC I structure
Carbonic anhydrase I	CA I	Carbonic anhydrase involved in acid-base balance regulation and CO ₂ transport (mucosa cell defense mechanism)
Carbonic anhydrase II	CA II	High-activity carbonic anhydrase essential for acid-base homeostasis and pH regulation (mucosa cell defense mechanism)
Carbonic anhydrase III	CA III	Muscle-specific carbonic anhydrase with antioxidant properties and cytoprotective functions (mucosa cell defense mechanism)
Cluster of differentiation 1d	CD1d	Antigen-presenting molecule that presents lipid antigens to natural killer T cells
Cluster of differentiation 3 gamma/3 delta	CD3 γ /3 δ	Subunit of CD3 complex essential for T-cell receptor signaling and activation
Cluster of differentiation 8	CD8	Co-receptor for MHC class I molecules on cytotoxic T cells involved in immune defense
Cluster of differentiation 161	CD161	Marker and regulator of natural killer cells and subsets of T cells
Cadherin-1 (E-cadherin gene)	CDH1	Cell adhesion protein critical for maintaining epithelial integrity and barrier function
Collagen type I alpha 1 chain	COL1A1	Major component of type I collagen involved in tissue structure and wound healing
Cornulin	CRNN	Epithelial differentiation marker involved in mucosal barrier integrity and response to stress
Connective tissue growth factor	CTGF	Growth factor that promotes fibroblast proliferation, adhesion, and extracellular matrix production
Decorin	DCN	Proteoglycan that regulates collagen fibrillogenesis and modulates growth factor activity
Early growth response protein 1	EGR-1	Transcription factor involved in cellular growth, differentiation, and stress responses
Extracellular signal-regulated kinase	p-ERK	Kinase in MAPK pathway involved in cellular proliferation, differentiation, and response to stress and inflammation
Fibroblast growth factor 2	FGF-2	Growth factor that stimulates cell proliferation, tissue repair, and angiogenesis
Fibronectin 1	FN1	Key extracellular matrix glycoprotein mediating cell adhesion, migration, wound healing, and fibrosis
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Glycolytic enzyme; commonly used housekeeping gene; involved in glycolysis, apoptosis, and nuclear functions
Hyaluronan synthase 2	HAS2	Enzyme-synthesizing hyaluronic acid; critical in extracellular matrix formation, tissue hydration, and repair
Heat shock protein 70	Hsp70	Chaperone protein that protects cells from stress and assists protein folding
Interleukin 1 beta	IL-1 β	Pro-inflammatory cytokine-mediating acute-phase responses and inflammation
Interleukin 6	IL-6	Cytokine with pro-inflammatory and anti-inflammatory roles; mediates fever and acute-phase responses
Interleukin 8	IL-8	Chemokine attracting neutrophils and other leukocytes to inflammatory sites, critical in acute inflammatory responses
Interleukin 18	IL-18	Pro-inflammatory cytokine that enhances cell-mediated immunity
Immunological critical value	ImmunoCRIT	Quantitative measure of immune cell populations, calculated as Treg/T-cell ratio indicating immune regulation status
Cellular proliferation marker	Ki-67	Nuclear protein expressed during active phases of cell division, a widely used proliferation marker
Keratin 14	KRT14	Structural protein in basal epithelial cells providing mechanical stability
Lysosomal cysteine protease	Cathepsin B	Protease involved in protein degradation, apoptosis, inflammation, and matrix remodeling

TABLE 2 (Continued)

Molecules and Outcomes	Abbreviation	Definition/Role
O-6-Methylguanine-DNA methyltransferase	MGMT	DNA repair enzyme that removes alkyl groups from the O ⁶ position of guanine
Major histocompatibility complex class I	MHC I	Cell surface proteins that present peptides from inside the cell to cytotoxic T cells
Major histocompatibility complex class II	MHC II	Cell surface proteins that present peptides from outside the cell to helper T cells
Matrix metalloproteinase 1	MMP1	Collagenase that degrades extracellular matrix components in tissue remodeling
Matrix metalloproteinase 2	MMP2	Gelatinase involved in extracellular matrix degradation and tissue remodeling
Matrix metalloproteinase 3	MMP3	Stromelysin that degrades multiple extracellular matrix components
Matrix metalloproteinase 7	MMP7	Matrilysin involved in extracellular matrix degradation and activation of other MMPs
Matrix metalloproteinase 9	MMP9	Gelatinase involved in extracellular matrix degradation and tissue remodeling
Matrix metalloproteinase 14	MMP14	Membrane-type MMP involved in extracellular matrix degradation and activation of other MMPs
Mucin 1	MUC1	Transmembrane mucin glycoprotein forming protective barriers on epithelial surfaces (laryngeal cell defense mechanism)
Mucin 2	MUC2	Secreted gel-forming mucin primary in intestinal mucus
Mucin 3	MUC3	Membrane-bound mucin in intestinal epithelia
Mucin 4	MUC4	Membrane-associated mucin with roles in cell signaling and lubrication
Mucin 5AC	MUC5AC	Secreted gel-forming mucin predominant in airway and gastric mucus
Mucin 5B	MUC5B	Secreted gel-forming mucin found in respiratory and salivary mucus
Mucin 6	MUC6	Secreted mucin involved in gastric mucosal protection
Mucin 7	MUC7	Secreted mucin found in saliva with antimicrobial properties
8-Hydroxy-2'-deoxyguanosine	8-OHdG	Biomarker of oxidative DNA damage and oxidative stress
Phosphorylated H2A histone family member X	p-H2AX	Phosphorylated histone H2AX, a sensitive marker of DNA double-strand breaks and genomic instability
Prostaglandin-endoperoxide synthase 2	PTGS2 (COX-2)	Enzyme catalyzing prostaglandin synthesis; plays a critical role in inflammation, pain, fever, and mucosal defense
Reactive oxygen species	ROS	Reactive molecules containing oxygen that participate in cellular signaling and oxidative stress
Sphingosine-1-phosphate	S1P	Bioactive sphingolipid mediator involved in cell growth, survival, and immune function
Sphingosine-1-phosphate receptor 1	S1PR1	Receptor for S1P that regulates cellular responses to this lipid mediator
Sphingosine kinase 1	Sphk1	Enzyme that catalyzes the phosphorylation of sphingosine to form S1P
Transforming growth factor beta 1	TGFβ-1	Growth factor with complex roles in cell proliferation, differentiation, and immune regulation
Tumor necrosis factor alpha	TNFα	Pro-inflammatory cytokine involved in systemic inflammation and acute-phase reaction
Regulatory T cell	Treg	Subset of T cells that modulate the immune system and maintain tolerance to self-antigens
Treg-specific demethylated region	TSDR	Specific DNA region demethylated in regulatory T cells, critical for stable Treg cell differentiation and function
Vascular endothelial growth factor A	VEGFA	Key regulator of angiogenesis and increased vascular permeability
Beta-catenin	β-Catenin	Dual-function protein involved in cell-cell adhesion and gene transcription
Caspase-1	Caspase-1	Protease enzyme activating pro-inflammatory cytokines (IL-1β, IL-18), a key mediator of pyroptotic cell death and inflammation

TABLE 3.
Summary of Key Findings

Outcomes	N	Primary Findings	References
<i>Histopathological Findings</i>			
Reduction of cell junction proteins	4	E-cadherin expression was reduced in pepsin-exposed tissues	14,16,21,33
Intercellular spaces dilatation	2	Reflux patients reported high intercellular space dilatation	19,33
Direct or indirect demonstration of immune cell infiltrate	5	Increased in basal and luminal layers of reflux laryngeal tissues	16,17,20,23,28
<i>Mucosa-Protective Mechanisms</i>			
Carbonic anhydrase expression	6	Studied in vocal fold and laryngeal tissue	13-16,27,29
Carbonic anhydrase type III expression	4	Reduced by pepsin exposure in mucosa of sLPRD patients	14-16,27
MUC expression	3	Reduced in sLPRD patients	14,22,26
<i>Mucosa Injury Mechanisms</i>			
Cell/DNA injuries/inflammation and pepsin detection	4	Pepsin is detected in the intracellular compartment of laryngeal cells	16,18,25,31
Oxidative stress mediators	2	Associated with pepsin-related laryngeal tissue injuries	31,34
<i>Molecular Findings</i>			
Expression of inflammatory mediators	2	Inflammatory mediator release is correlated with pepsin injury	17,34
Expression of matrix metalloproteinases	2	Several MMP expressed in pepsin-exposed tissues	30,33

depletion being related to pepsin exposure (vocal fold^{15,16} and ventricular bands¹⁵), and associated with clinical symptoms.¹⁴ This depletion was confirmed by two other teams in posterior commissure and vocal fold tissues of suspected LPRD patients versus controls.^{16,27} Only Wood et al did not demonstrate significant differences between suspected LPRD and controls, but controls consisted of patients with LPRD symptoms and RSI < 12 and RFS < 6.²⁹

MUC are a family of molecules protecting the mucosa through the secretion of a hydrated mucus. Johnston et al observed downregulation of MUC4 and 5AC expression in suspected LPRD compared with controls,¹⁴ while Samuels et al reported similar findings for MUC2, 3, 5AC, and 5B.²² El-Sayed et al reported downregulation of MUC2 in posterior commissure biopsies, and MUC5AC in vocal fold and ventricle tissues of suspected LPRD patients compared with controls. Similarly to CA expression, Wood et al did not observe significant vocal fold differences in MUC1,2,3B,4,5B,6 and 7 between RSI > 12-RFS > 6 patients and those with laryngopharyngeal symptoms and findings without positive RSI and RFS.²⁹ However, in arytenoid and posterior commissure biopsies, these authors reported downregulation of MUC5B in suspected LPRD patients compared to others.²⁹

Experimental model heterogeneity

There were substantial heterogeneities across studies for inclusion criteria, reflux diagnosis, anatomical laryngeal regions, analysis approaches, and outcomes. There was no

study considering the inclusion of patients with a demonstrated LPRD at the 24-hour HEMII-pH. Single- or dual-probe pH metry was used in two^{16,19} and three^{15,21,25} studies, respectively. Jetté et al based the LPRD diagnosis on MII-pH without pharyngeal sensor.²⁸ In other studies, patients were selected with RSI and/or RFS using validated (RSI > 13-RFS > 7)^{27,35} or unvalidated thresholds.^{20,22,23,29} The selection criteria of patients with LPRD were not clearly provided in eleven studies.^{13,14,17,18,24,26,30-34} Similar findings were observed for the control groups that reported a myriad of inclusion and exclusion criteria. Notably, the control groups in several studies consisted of patients with laryngopharyngeal symptoms and clinical findings who did not meet established RSI/RFS thresholds or pH-metry criteria for classification as suspected LPRD patients.^{21,29} The anatomical laryngeal regions selected for biopsies represent an additional source of heterogeneity across studies.

DISCUSSION

The development of experimental research investigating the enzyme-mediated epithelial alterations in vocal fold tissues is a key approach to better understand the relationship between dysphonia and LPRD. Despite the expanding of research in this field since the 2016 review of LPRD-associated dysphonia mechanisms,² a definitive mechanistic relationship between epithelial microalterations and objective voice quality deterioration remains scientifically unestablished.

The findings of the present review demonstrate that laryngeal, particularly vocal fold, biopsies of patients with

suspected LPRD reveal a substantial number of histological and functional alterations, including inflammatory cell infiltration,^{16,17,20,23,28} cell junction proteolysis,^{14,16,21,33} intercellular space dilatation,^{19,33} pepsin-induced cell DNA damage, and increases in oxidative stress mediators and tissue injuries.^{31,34} While some tissue lesions are nonspecific, refluxate pepsin can significantly increase the occurrence of tissue injury through the reduction of certain markers of epithelial defense mechanisms, including mucin genes and CAs.

In clinical practice, it has been well-established that numerous benign vocal fold lesions—including nodules, polyps, and leukoplakia—are favored by the occurrence of repetitive microtraumas to the vocal fold epithelium,³⁸ with LPRD representing a significant etiological cofactor.⁴ Although no investigation has yet established a definitive mechanistic relationship between vocal fold epithelial alterations and objective voice quality impairments, the molecular pathway observations of two recent studies suggested a potential role of pepsin in the pathogenesis of leukoplakia.^{32,35} Thus, the findings of this systematic review provide a foundational framework for designing future experimental investigations aimed at elucidating the pathophysiological mechanisms underlying benign vocal fold lesion development.

The voice quality is influenced by the regenerative capacity of vocal fold tissue in response to chemical (tobacco constituents, pollution, and digestive enzymes), infectious (viral or bacterial laryngitis), or mechanical (longitudinal/transversal mucosal stress during phonation) aggressions. Adequate tissue healing maintains the intrinsic biomechanical and viscoelastic properties of the vocal folds, which are essential for optimal phonatory function. In this systematic review, some investigations confirm that pepsin exposure significantly impairs epithelial defense mechanisms and disrupts wound healing processes in vocal fold tissue,^{14–16,22,26,27} corroborating the findings of Roh et al demonstrating pepsin-mediated vocal fold healing alterations in an animal model.^{37,39}

Despite considerable histopathological evidence demonstrating pepsin-induced ultrastructural changes in vocal fold tissues across studies in this systematic review, several critical methodological limitations—including non-validated LPRD diagnostic methods, inconsistent biopsy sampling techniques, and heterogeneous control group selection—substantially limit the drawing of definitive conclusions regarding pathophysiological mechanisms.

First, the primary limitation was the absence of HEMII-pH for establishing the LPRD diagnosis across all studies. The 24-hour HEMII-pH is considered as the gold standard diagnostic approach¹ for detecting acid, weakly acid, and alkaline pharyngeal reflux events.⁴⁰ Clinical studies demonstrated that the majority of LPRD patients report weakly acid or alkaline reflux events,^{41–43} making insufficient the pH monitoring device.^{1,41} The utilization of pH monitoring to detect esophageal or pharyngeal acid reflux as an inclusion criterion may introduce selection

bias, predominantly recruiting patients with acid-predominant LPRD while excluding those with weakly acidic or non-acidic reflux patterns. In the same vein, experimental studies have largely overlooked other digestive enzymes in the investigation of pathophysiological mechanisms underlying the LPRD-related vocal fold mucosa alterations. A few studies suggested that bile salts, elastase, and trypsin could play a key role in the development of LPRD symptoms and findings, with most of these enzymes demonstrating optimal activity in weakly acidic or alkaline pH environments.^{44–46} In 2006, Sacco et al detected elastase in bronchoalveolar fluid of children with GERD at the GI endoscopy, strengthening the important role of elastase in the development of respiratory diseases associated with reflux.⁴⁷

Second, the consideration of patients with laryngopharyngeal symptoms and findings, but negative RSI and RFS scores, is another limitation of some studies.²⁹ Indeed, RSI and RFS are nonspecific clinical instruments, which do not include all symptoms and signs associated with LPRD.^{48,49} In this context, their use for determining patients as LPRD or not can consist of an inclusion bias.

Third, despite widespread recognition of the critical importance of precise anatomical localization and corresponding site-specific molecular expression patterns across investigations, inadequate documentation of biopsy anatomical sites in several studies substantially limits the generalizability of their findings. The inclusion of cell biopsies from several laryngeal anatomical sublocations is another potential heterogeneity factor because, depending on the laryngeal regions, tissues undergo different types of mechanical forces, with the free edge versus the posterior larynx being dramatically different. Moreover, some confounding factors of laryngitis have not been considered in most studies, including tobacco consumption, alcohol (posterior commissure tissue), pollution (eg, microplastics), and microbiome differences across patients regarding their comorbidities. Microbiome can be important for future studies because recent studies suggested that it should be involved in the development of laryngeal benign lesions of the vocal folds, reflux laryngitis, and laryngeal squamous cell carcinoma.^{50,51} The etiology of the measured outcomes could be therefore attributed to multiple factors, not LPR alone. Subsequent investigations should systematically address potential confounding variables implicated in vocal fold ultrastructural alterations, including pollution, vocal hygiene practices, phonatory behavior patterns, and tobacco exposure—the latter being inadequately controlled for in current studies.

CONCLUSION

Specimens from laryngeal and vocal fold biopsies in patients with suspected LPRD demonstrate substantial histopathological, functional, and molecular alterations. Pepsin-mediated inhibition of mucosal defense mechanisms exacerbates tissue injury and impairs healing processes.

Future investigations should examine additional digestive enzymes' contributions to vocal fold alterations while correlating findings with objective voice quality parameters.

Author Contributions

Guanjin Chen: Contributions: design, acquisition of data, data analysis and interpretation, drafting, final approval, and accountability for the work; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. **Jerome R. Lechien:** Contributions: design, acquisition of data, data analysis and interpretation, drafting, final approval, and accountability for the work; final approval of the version to be published; agreement to be accountable for all aspects of

the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of Competing Interest

The authors have no financial interest in the subject under discussion. All authors have read and approved the paper. Would you be so kind to consider the present paper and send us the reviewer's comments.

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Appendix 1: *In Vitro* Studies

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Ylitalo and Thibeault, 2006 ¹⁷	Prospective	Ventricles (a), post. com (b)	RT-PCR	<i>mRNA expression of fibro-blasts (a/b)</i>	<i>pH/pepsin/time effect</i>
	Uncontrolled	Exposed to pepsin + pH 4 or 5		ATF-3, TGFβ-1, VEGF, and CTGF(a)	pH(S)
		During 10, 30, 60, and 240 seconds		MMP1, MMP-2, Decorin, and EGR-1(a)	pH(S)
				TGFβ-1, FGF-2, CTGF, and MMP1(b)	pH(S)
				ATF-3(a)/ATF-3, VEGF(b) ATF-3, CTGF, and MMP1(a) TGFβ-1, FGF-2, CTGF, MMP1-2, and Decorin(b) FGF-2(a)/EGR-1(b)	Pepsin(S) Time effect(S) Time effect(S) NS
Johnston et al, 2007 ¹⁸	Prospective	Post. com: 2, post. cricoid area: 2	Microscopy	Intracellular pepsin	+
McCann et al, 2020 ³⁰	Uncontrolled	Exposed to human pepsin			
	Prospective	Human immortalized vocal fold	qPCR	MMP9, FN1, and COL1A1	Gr3 > Gr6
	Controlled	Fibroblasts exposed to 24/48 hours	(expression)	MMP1, HAS2, and CDH1	Gr3 = Gr6
		Gr1: pepsin (0.1 mg/ml, pH = 7)		MMP1,9, FN1, COL1A1, HAS2, and CDH1	Gr1 = Gr6, Gr2 = Gr6
		Gr2: pepsin (1.0 mg/ml, pH = 7)		MMP1, FN1, HAS2, Gr4=Gr6 and CDH1	
Ao et al, 2022 ³²	Prospective	Gr3: TGF-β (5 ng/ml, pH = 7)		MMP9, COL1A1	Gr6 > Gr4 (48 hours)
		Gr4: pepsin (0.1 mg/ml, pH = 5)		HAS2	Gr5 > Gr6
		Gr5: pH = 5-Gr6: pH = 7	ELISA	MMP9, fibronectin	Gr1, Gr2, Gr3 = Gr6 Gr6 > Gr4, Gr5
					Gr4 > Gr3 > Gr2 > Gr1, Gr1 > Gr5
	Controlled	Gr1: nothing	Transwell assay Wound healing CCK-8	Migration, proliferation Proliferation Apoptosis	Gr7, Gr8 > Gr1 Gr1 > Gr2 > Gr3 > Gr4 Gr5 > Gr1, Gr1 > Gr7, Gr8

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Samuels et al, 2023 ³³	Prospective	Gr6: fluorescent framework Gr7, 8: Tu212-AMC-HN-8 Vocal fold cells	Microscopy	Cell dissociation	Gr2 > Gr3 > Gr4
	Controlled	Gr1: pH = 4 Gr2: pepsin (1mg/l, pH = 4) Gr3: pepsin+1-ul APR (pH = 4) Gr4: pepsin+10-ul APR (pH = 4) Gr5: pepsin+100-ul APR (pH = 4) Gr6: pepsin+1-ul FOS (pH = 4) Gr7: pepsin+10-ul FOS (pH = 4) Gr8: pepsin+20-ul GM6001 (pH = 4) Gr9: CT (pH = 7.4)	WB Immuno fluorescence RT-qPCR	Cleavage of E-cadherin MMP1,3,7,9,14 MMP14 MMP1,9,14 MMP7	Gr2 > Gr6 > Gr7 Gr2 > Gr4 > Gr1 = Gr9 Gr2 > Gr8 > Gr1 = Gr9 Gr2 > Gr9 Gr1 > Gr9 Gr2 > Gr4 > Gr9 Gr2 > Gr5 > Gr9

One study reported *in vitro* findings from laryngeal mouse cells.³² Molecule abbreviations are described in Table 2. Abbreviations: AGJ+p, artificial gastric juice + pepsin; IHC, immunohistochemistry; NP, not provided; NS, nonsignificant; post.com, posterior commissure; RT-PCR, reverse transcription-polymerase chain reaction; S, significant; WB, Western blot.

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