The Role of Laryngopharyngeal Reflux Disease in Laryngeal and Hypopharyngeal Squamous Cell Carcinoma: A Systematic Review

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SUMMARY: Background. Laryngeal and hypopharyngeal squamous cell carcinomas (L/HSCC) are common head and neck cancers with rising incidence globally. Laryngopharyngeal reflux disease (LPRD) is suspected to play a role in carcinogenesis through unknown mechanisms. The aim of this review was to summarize the literature findings about the potential carcinogenesis mechanisms associated with LPRD in L/HSCC.

Methods. A PubMed, Embase, and Web of Science database search was carried out by two independent investigators for studies investigating laryngeal and hypopharyngeal mucosa injuries and premalignancy modifications related to LPRD refluxate. The authors considered clinical and experimental studies using human biopsies, animal models, and cell lines that investigated potential mechanistic associations between LPRD and L/HSCC progression. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements were followed.

Results. Of the 689 identified papers, 34 studies were included. There were human tumor tissue studies (n = 8), human cell line studies (n = 19), and animal models (n = 12). Across methodologies, consistent evidence supported reflux-induced NF-κB pathway activation, oncogenic miRNA dysregulation, epithelial-mesenchymal transition, abnormal cell proliferation and differentiation, increased DNA damage, imbalance in the expression of tumor-promoting and tumor-suppressing genes, and altered cellular stress responses. Both pepsin and bile acids demonstrated carcinogenic potential through multiple molecular pathways, despite significant methodological heterogeneity across studies.

Conclusion. LPRD-refluxed pepsin and bile acids may substantially contribute to the carcinogenesis of laryngeal and hypopharyngeal cells through several pathways. Future translational studies documenting refluxed gastroduodenal enzymes in tumor samples are needed to better determine their role in the development of L/HSCC through understanding the underlying biomolecular cellular mechanisms.

Key Words: Carcinoma–Reflux–Otolaryngology–Head Neck Surgery–Larynx.

INTRODUCTION

Laryngeal and hypopharyngeal squamous cell carcinomas (L/HSCC) are two prevalent head and neck malignancies corresponding to 5.3% and 0.4% of all cancers, respectively.[1–3] Laryngeal squamous cell carcinoma (LSCC) is the second most common head and neck cancer, accounting for 211,000 new cases and 126,000 deaths yearly worldwide.[1,2] Hypopharyngeal squamous cell carcinoma (HSCC) accounted for 84,254 new cases in 2020, and the number of deaths was 38,599 worldwide.[3] Both cancers reported similar major risk factors, including prolonged tobacco and alcohol use (supraglottic), Plummer-Vinson syndrome, asbestos exposure, reflux, and betel nut chewing.[4–6] The association between L/HSCC and reflux remains not established due to the lack of high-quality clinical studies considering laryngopharyngeal reflux disease (LPRD) rather than gastroesophageal reflux disease (GERD), objective evaluations of LPRD, and gastroduodenal enzyme patterns in laryngopharyngeal tissues, leading to the controversial results of meta-analyses.[7–9]

Regarding the lack of clinical studies considering the identification of LPRD through 24-hour hypopharyngeal-esophageal multichannel intraluminal impedance-pH testing (HEMII-pH),[10] and the tumor/laryngopharyngeal fluid measurements of gastroduodenal enzymes,[11] in patients with L/HSCC, the investigation of biomolecular and cellular mechanisms underlying the enzyme-induced development of premalignant mucosa lesions may provide relevant findings for understanding the relationship between reflux and L/HSCC.

The aim of this review was to summarize the literature findings about the potential carcinogenesis mechanisms associated with LPRD in L/HSCC.

MATERIALS AND METHODS

This review was conducted by two independent investigators (G.J.C. and J.R.L.) following the Preferred Reporting Items for Systematic Reviews and Meta-

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Analyses (PRISMA) checklist.[12] The criteria for considering studies were based on population, intervention, comparison, outcome, timing, and setting (PICOTS) framework.[13]

Types of studies

From March to July 2025, the literature search included prospective, retrospective, or cross-sectional studies published between 1990 and March 2025 in English-language peer-reviewed journals. Studies investigated the relationship between LPRD, refluxed enzymes, and the premalignant modifications of laryngeal and hypopharyngeal epithelial cells/mucosa using human samples, animal models, or *in vitro* approaches. Studies investigating the clinical findings of reflux, including GERD and LPRD findings, without consideration of mucosa macro- or microscopic changes, were excluded.

Population

For studies using human population samples, the inclusion criteria used for the reflux disease diagnosis were extracted. The diagnosis of LPRD was confirmed for patients with more than one hypopharyngeal reflux event at the 24-hour hypopharyngeal-esophageal multichannel intraluminal impedance-pH monitoring (HEMII-pH).[14,15] The use of oropharyngeal pH monitoring, dual- or triple-probe pH monitoring with pharyngeal pH sensor supported the acidic LPRD/GERD diagnoses but did not confirm the LPRD diagnosis regarding the lack of pharyngeal impedance system.[14,15] Patients who were selected with reflux validated patient-reported outcomes questionnaires and/or validated sign instruments were considered as suspected LPRD patients. Patients meeting the Montreal[16] or Lyon[17] consensus criteria were considered as GERD patients.

Outcomes

Data from *in vitro* cell line experiments and *in vivo* studies were extracted by two independent investigators. The primary outcomes consisted of data describing potential associations or mechanisms of action between LPRD, refluxed enzymes, and laryngeal and hypopharyngeal mucosal morphological and functional changes considered as premalignant steps. The results were organized into three main categories based on the experimental models: (1) human tumor tissue studies, which investigated molecular and cellular alterations in surgically resected specimens; (2) human cell line studies, which examined the effects of reflux components (pepsin and bile acids) on laryngeal and hypopharyngeal epithelial cells; and (3) animal model studies, which explored the carcinogenic potential of reflux contents *in vivo*. Within each category, we specifically analyzed several key pathways and mechanisms, including immune responses; pepsin expression; activation of oncogenic signaling pathways (particularly NF-κB); expression of stress proteins; epithelial-mesenchymal transition markers; cell proliferation and viability; and microRNA dysregulation. The morphological changes assessed included impaired cell proliferation and differentiation, histopathological abnormalities, and cell DNA damage. Functional cellular characteristics included alterations in inflammatory cell mediators, expression of tumor-promoting and tumor-suppressing genes, and oxidative stress biomarkers. The reflux parameters assessed included the features of reflux events (esophageal/pharyngeal events, liquid/gas/mixed, and pH), the detected digestive enzymes (pepsin, bile salts, trypsin, lipases, and elastase), and their respective methods of detection (eg, IHC, ELISA). The timing of these effects ranged from immediate cellular responses to long-term preneoplastic and neoplastic transformations, while the setting encompassed both acidic and nonacidic environments to simulate various reflux conditions.

Intervention and comparison

Intervention may consist of application of reflux/gastroduodenal content onto laryngeal and hypopharyngeal cells/tissue. Evolution of mucosal morphology, histology, and functioning from pre- to post-treatment of LPRD was similarly considered.

Time and setting

There were no strict criteria for time and setting.

Search strategy

The literature search was conducted by the two independent investigators on PubMed, Embase, and Web of Science databases for relevant peer-reviewed publications on laryngeal and hypopharyngeal premalignant/malignant features related to LPRD. The following MeSH terms were used for the search strategy: larynx; hypopharynx; neoplasms; carcinoma; gastroesophageal reflux; bile acids and salts; pepsin; trypsin; microbiome. The non-MeSH term "laryngopharyngeal reflux" was added to the key words. 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

A total of 4430 publications were found from the following electronic databases: Embase (n = 1951), PubMed (n = 1762), and Web of Science (n = 717). After removing 725 duplicates across databases, 3705 records underwent initial screening (Figure 1). Title and abstract assessment excluded 3016 records that did not meet the inclusion criteria. The remaining 689 full-text articles were carefully evaluated, resulting in the inclusion of 34 studies investigating the potential mechanistic links between LPRD and carcinogenesis of L/HSCC.[18–51] Of the 34 studies, some studies reported human, cell lines, and/or

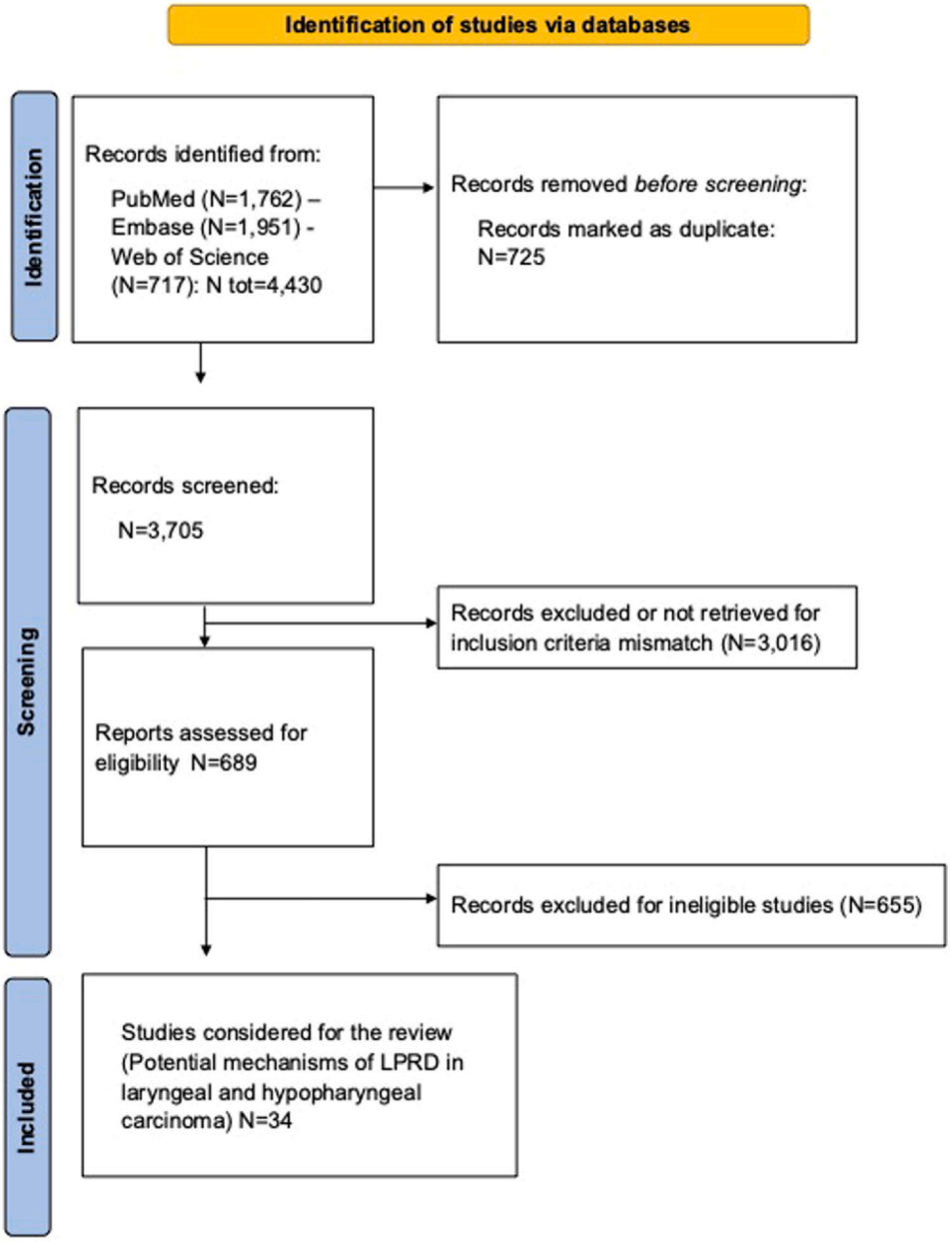

FIGURE 1. Chart flow. LPRD, laryngopharyngeal reflux disease.

animal data. There were human tumor tissue studies (n = 8, Table 1) focused primarily on analyzing molecular and cellular alterations within surgically resected LSCC (n = 5)[18–22] or HSCC (n = 3)[23–25] specimens. Human cell line studies (n = 19, Table 2)[18,22,23,25–39] consisted of *in vitro* models derived from human laryngeal or hypopharyngeal epithelial cells. Six studies[22,23,26,27,39,51] involved laryngeal cell lines exposed to pepsin (n = 4)[22,23,27,39] or bile acids (n = 1),[26] while studies with hypopharyngeal cell lines (n = 15) focused on the effects of pepsin (n = 7 studies)[18,23,25,27,28,30,37] or bile acids (n = 8 studies).[29,31–36,38] Among the 12 animal model studies[18,40–50] (Table 3), one study[18] was based on a porcine laryngeal model, while 11 studies[40–50] consisted of murine models of laryngeal (n = 3)[40,42,49] or hypopharyngeal specimens (n = 8).[41,43–48,50] A summary of LPRD cell pathway is found in Figure 2.

Human tumor tissue studies

Among the eight studies analyzing human tumor specimens, two studies explored immune responses of laryngeal mucosa exposed to reflux stimuli. The authors observed an increased CD8+ T-cell expression,[20] and aberrant MHC molecule expression.[19] Two studies found significantly higher pepsin expression in laryngopharyngeal tumor tissues compared with healthy controls,[23,25] with a positive correlation of pepsin expression with lymph node metastasis.[25] Bao et al found significantly higher proton pump (H^+/K^+-ATPase) expression in malignant laryngeal tissues compared with paracarcinomatous and normal tissues.[21] In

TABLE 1.
Human Tissue Studies

References	Design	LPRD Diagnosis	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Johnston, 2006 (18)	Prospective Controlled	24-hour dual-probe pH metry	Human laryngeal tissue Gr1: healthy (N = 6) Gr2: LPR and laryngeal lesions (N = 19) Gr3: LPR and laryngeal cancer (N = 6)	WB	sept-70 sept-53 Hsp70	Gr1 > Gr3, Gr1 > Gr2 Gr1 > Gr3, Gr1 > Gr2 (NS) Gr1 > Gr3, Gr1 = Gr2
Birchall, 2008 (19)	Prospective Controlled	RSI > 21	Human laryngeal samples LPR patients (N = 12) Healthy (N = 11)	IHC	Expression CD161, MHC β2m MHC I, II MHC CD1d	 Gr1 > Gr2 Gr1 = Gr2 Gr1 > Gr2
Rees, 2008 (20)	Prospective Controlled	RSI > 21	Human laryngeal samples *Posterior vocal folds tissue* Gr1: LPR (N = 12) Gr2: CT (N = 11)	IHC	Cells' infiltration B cells, neutrophils Eosinophils, monocytic CD8 lymphocytes (luminal and basal layers) Expression of MHC I, II β2-microglobulin	 Gr1 = Gr2 Gr1 = Gr2 Gr1 > Gr2 Gr1 = Gr2 Gr1 > Gr2
Johnston, 2012 (23)	Prospective Controlled	RSI > 8 and RFS > 4	Human laryngopharynx samples Posterior cricoid biopsy Gr1: laryngopharynx cancer (N = 5) Gr2: healthy CT (N = 5)	WB	The presence of pepsin	Gr1 > Gr2
Sasaki, 2019 (24)	Prospective Controlled	Endoscopy History of GERD	Human hypopharynx samples Human HSCC specimens (N = 8) Gr1 = with bile reflux (N = 3) Gr2 = without bile reflux (N = 5) Gr3: adjacent normal tissue	Histological Analysis IHC qPCR	p-NF-κB NF-κB-related mRNA RELA, c-REL, BCL-2, EGFR, WNT5A, and ΔNp63, TNF-α, IL-1B, IL-6, and STAT3 PKI3CA, NOTCH1, NOTCH2, NOTCH3, and TP53 miR-21, -155, and -192 miR-34a,-375, and -451a miR-451a miR-21/miR-375 miR-489, miR-504, and miR-99a	Gr1 > Gr2, Gr1 > Gr3 Gr1 > Gr2, Gr1 > Gr3 Gr1 > Gr2, Gr1 > Gr3 Gr1 > Gr2, Gr1 > Gr3 Gr1 = Gr2 = Gr3 Gr1 > Gr2, Gr1 > Gr3 Gr2 > Gr1, Gr3 > Gr1 Gr2 = Gr3 Gr1 > Gr2, Gr1 > Gr3 Gr2 > Gr1

TABLE 1 (*Continued*)

References	Design	LPRD Diagnosis	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Bao, 2020	Prospective	N.P	Human larynx samples	IHC	H+/K+-ATP α-subunit	Gr1 > Gr2,Gr3
(21)	Controlled		Gr1: larynx cancer tissue (N = 30)	RT-PCR	H+/K+-ATP β-subunit	Gr1 > Gr2,Gr3
			Samples: N = 90	and WB		
			Gr2: para-larynx cancer tissue (N = 20)		Correlation	
			Samples: N = 60		Subunit α/β and clinical features	NS
			Gr3: normal larynx tissue (N = 59)		Subunit α/β and pathological features	NS
			Samples: N = 195			
Niu, 2020	Prospective	RFS, RSI	Human hypopharynx samples	IHC	Pepsin expression	Gr1 > Gr2
(25)	Controlled		HPSCC patients' biopsy (N = 70)		Correlation (pepsin)	
			Gr1: HPSCC (N = 70)		and nodal involvement	S
			Gr2: HPSCC adjacent tissue (N = 70)		and alcohol, tobacco	NS
			Gr3: stomach tissues *with esophageal cancer*		and tumor stage, grade	NS
			Gr4: tonsil tissues (N = 4)			
Tan, 2019	Prospective	N.P	Laryngeal carcinoma tissue (N = 87)	IHC	Correlations: IL-8 and pepsin	Significantly positive
(22)	Controlled		Gr1: pepsin positive (N = 65)		β-catenin, vimentin, and pepsin	Significantly positive
			Gr2: pepsin negative (N = 22)		E-cadherin and pepsin expression	Significantly negative

Abbreviations: β2m, beta-2 microglobulin; BCL-2, B-cell lymphoma 2; β-catenin, beta-catenin; CD, cluster of differentiation; c-REL, proto-oncogene c-Rel; CT, control; ΔNp63, delta Np63 (N-terminally truncated p63); E-cadherin, epithelial cadherin; EGFR, epidermal growth factor receptor; GERD, gastroesophageal reflux disease; HSCC, hypopharyngeal squamous cell carcinoma; Hsp70, heat shock protein 70; IHC, immunohistochemistry; IL-1B, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; LPR(D), laryngopharyngeal reflux (disease); MHC, major histocompatibility complex; miR, microRNA; NF-κB, nuclear factor kappa light-chain enhancer of activated B cells; NOTCH, notch receptor; NS, not significant; p-NF-κB, phosphorylated nuclear factor kappa B; PKI3CA, phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit alpha; qPCR, quantitative polymerase chain reaction; RELA, v-rel avian reticuloendotheliosis viral oncogene homolog A; RFS, reflux symptom index; RSI, reflux symptom index; RT-PCR, reverse transcription polymerase chain reaction; S, significant; sep, stress-exposed protein; STAT3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor alpha; TP53, tumor protein p53; Vimentin, intermediate filament protein; WB, Western blot; WNT5A, Wnt family member 5A.

TABLE 2.
Cell Line Studies

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Johnston, 2006	Prospective	HPSCC FaDu	WB	Pepsin expression in cells	NS (Gr1)
(18)	Experimental	Exposed to pepsin-TRITC for 1 h	Confocal		NS (Gr2, in 30 min)
		Gr1: without human pepsin, 3 h	microscopy		S (Gr2, after 60 min)
		Gr2: after human pepsin, 3 h			
Tan, 2019	Prospective	TU212, Hep-2(a,b) cell lines	Microscopy	Cell proliferation	Gr1 > Gr3,Gr4
(22)	Experimental	Exposed to pepsin, 2 h, pH 7	Transwell migration	Cell migration, S phase	Gr1 > Gr3,Gr4
	Controlled	Gr1: pepsin+	Flow cytometry	IL-6,10,1β,12p70, TNF	Gr1 = Gr2,Gr4
		Gr2: pepsin + pepstatin A	qRT-PCR	E-cadherin	Gr2,3,4 > Gr1
		Gr3: pepsin + IL-8 inhibitor/	WB	Vimentin, β-catenin, and Il-8	Gr1 > Gr2,3,4
		Gr4: CT			
Johnston, 2012	Prospective	FaDu cells and laryngeal epithelial cells	Flow cytometry	Cells in S phase/proliferation	Gr1,4 > Gr3, dose dep
(23)	Experimental	exposed to	miRNA	84 genes related to cancer	Gr1: 3 up, 24 down
	Controlled	pepsin (0.01, 0.1, or 1 mg/ mL), pH 7, 1 h	SuperArrays	1.5-fold change	Gr2: 1 up, 30down
		Gr1: FaDu + pepsin		88 genes related to cancer	Gr1: up, 24 down
		Gr2: LEC + pepsin		1.5-fold change	Gr2: 9 up,15 down
		Gr3: CT		let miRNA	Gr3 > Gr2
		Gr4: FaDu + inactivated pepsin		Ras protein	Gr2 > Gr3
Niu, 2020	Prospective	HPSCC FaDu exposed to	Flow cytometry	Proliferation, S-phase cells	Gr1 > Gr2
(25)	Experimental	pig pepsin, 0.5,1, and 2 h pH 7	Immunofluorescence	Cells in G1 phase	Gr2 > Gr1
	Controlled	Gr1: pepsin	and WB	Cells in G2/M phase	Gr1 = Gr2
		Gr2: CT	PCR array	Lysosome co-localization	Gr1 > Gr2
				NF-κB p65, p21, c-Myc, and p-p65	Gr1 > Gr2
				TNF-α, BCL2A1, and p-IκB	Gr1 > Gr2
				TNFSF10, HES5	Gr2 > Gr1

TABLE 2 (*Continued*)

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Sung, 2003	Prospective	Human pharyngeal mucosa cells	WB	COX-2 expression	Gr1 > Gr4 CD dose dep
(26)	Experimental	*around the palatine tonsils*	RT-PCR	and COX-2 mRNA	Gr1 > Gr4 acidities dep
	Controlled	Exposed to chenodeoxycholate (CD)			Gr1 > Gr4 (in 2 min)
		Gr1: CD 20 h			Gr1 (Acid & CD > CD > Acid)
		CD = 50, 100, and 200 μmol/L, pH 6,7			Gr3 > Gr2 > Gr4
		Gr2: actinomycin-D + CD, Gr3: CD			
		CD = 100 μmol/L, pH 6, 10 min			
		Gr4: CT, pH 7			
	Prospective	Human larynx carcinoma cells	RT-PCR	and COX-2 mRNA	Gr1 (Acid & CD > CD > Acid)
	Experimental	SNU-1041	luciferase promoter assay	COX-2 promoter activity	Gr1 > Gr2
	Controlled	Gr1: CD200 μmol/L, 1 h; Gr2: CT, pH 7			
Samuels, 2013	Prospective	FaDu cells exposed to curcumin	Transmission electron microscopy	Mitochondrial damage	Gr4 > Gr1,2,3 abrogated
(27)	Experimental	ecabet sodium, anthocyanin-enriched	Cell count assay	Hyperproliferation	Gr4 > Gr1,2,3
	Controlled	black raspberry	Real-time PCR array	84 cycles' regulatory gene expression	Gr4: 3 up, 28 down
		Exposed to pepsin (0.1 mg/mL), pH 7, 1 h	Only in CM group	28 genes down in Gr4	Gr1: 26 negative
		Gr1 = CM (10 uM) + pepsin		3 genes up in Gr4	Gr1: 2 changed
		Gr2 = ES (125 ug/mL) + pepsin	In vitro kinetic assay	Peptic activity	Gr4 > Gr1,2,3 dose dep
		Gr3 = BRB (100 ug/mL) + pepsin			
		Gr4 = BSA + pepsin; Gr5: CT			
Kelly, 2014	Prospective	FaDu cells exposed to	Cell migration assay	RDW	Gr1 > Gr2 (a,b,a > b)
(28)	Experimental	pepsin 0.01, 0.1, and 1 mg/mL (a,b,c)	Apoptosis resistance	Cell viability	Gr1 > Gr2 (NS) (a,b,c)
	Controlled	24 h, 4 times over 2w, pH 7	Assay		
		Gr1: pepsin; Gr2: CT	Clonogenic assay	Colony-forming ability	Gr1 > Gr2 (b,c, b=c)

TABLE 2 (*Continued*)

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Sasaki, 2016	Prospective	Human hypopharynx samples	Immunofluorescence	Nucleus p65	Gr1, Gr2, Gr3, Gr5 > Gr4 (a,b)
(29)	Experimental	HHPCs, HHK, and UMSCC-11B (a,b,c)		Cytoplasmic Bcl-2	Gr1, Gr2, Gr3, Gr5 > Gr4 (a,b)
	Controlled	Exposed to	WB	p-p65/p65, p-IκB-α, and Bcl-2	Gr1 > Gr2, Gr3, Gr4 (b)
		bile salts (BA), 15 min, 3/d, 5d, pH 4.0, 7.2		p-IκB-α	Gr1 > Gr4(a,b), Gr5 > Gr4 (a)
		Gr1: acidic BA		Bcl-2	Gr1 > Gr4 (a,b)
		Gr2: neutral BA		Correlation	
		Gr3: acid control		Bcl-2 and p-IκB-α	S (a,b)
		Gr4: neutral control		Bcl-2 and p-p65	S (a,b)
		Gr5: USBA/deoxycholic acid/DCA	Luciferase assay	NF-κB transcriptional activity	Gr1, Gr2, Gr3 > Gr4, Gr6 (b)
		5 or 15 min, 7.2			Gr4, Gr6 = CT
		Gr6: glucose (33 mM)			Gr5 < CT (b)
		5 or 15 min, 5d	RT-qPCR	RELA, c-REL, Bcl-2, and TNF-α	Gr1 > Gr2, Gr3, Gr4 (a,b)
		Gr7: CT		TNF-α, EGFR, STAT3, WNT5A, ΔNp63,	Gr1 > Gr2, Gr3, Gr4 (a,b)
		# UMSCC-11B(C), no treatment	Luciferase assay	and Tp53	Gr4 > Gr1, Gr2 (a,b)
				p-p65, p-IκB-α, and Bcl-2	Up(S) in C
				NF-κB transcriptional genes	Up(S) in C
				Bcl-2, EGFR, WNT5A, and TNF-αGr1,	Gr1, Gr2 > CT (a,b)
				Bcl-2, NF-κB ,c-REL, and RELA	Gr1, Gr3 > CT (a,b)
				Bcl-2, EGFR, WNT5A, TNF-α, ΔNp63, and STAT3	Gr1 = Gr5 > CT (a,b)
				Tp53	Gr1, Gr2, Gr5 < CT (a,b)
Sasaki, 2016	Prospective	Human hypopharynx samples	Immunofluorescence	Nucleus p65	Gr1 = CT (a,b)
(30)	Experimental	HHPCs, HHK (a,b) exposed to	WB		Gr2 (a), Gr3 (b) > CT
	Controlled	pepsin 0.1, 0.05, and 0.01 mg/mL,		Total and nucleus p-p65	Gr4 > Gr1 (a,b)
		pH 4, 5, 7		and bcl-2 cytoplasmic/nucleus	Gr2 > Gr5 (a), Gr3 > Gr6 (b)
		Gr1: pepsin, pH 4		correlation	
		Gr2: pepsin, pH 5		p65 and p-IκB-α/p65 and Bcl-2	S (a)/NS (a)
		Gr3: pepsin, pH 7	Luciferase assay	NF-κB transcriptional activity	Gr1 = Gr2 = Gr3 (a,b)
		Gr4: pH 4			Gr2 > Gr5 (a), Gr3 > Gr6 (b)
		Gr5: pH 5			Gr2(b), Gr3(a) < CT
		Gr6: pH 7			NS in Gr7
		Gr7: inactivated pepsin	RT-qPCR	NF-κB (TFs), RELA, c-REL, bcl-2, TNF-α, EGFR, STAT3, wnt5α, and Tp63	Gr1 = CT (a,b)

TABLE 2 (*Continued*)

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
		Gr8: CT			Gr4 > Gr1, Gr5 > Gr2, Gr6 > Gr3 (a)
				Tp53	Gr1 > Gr4 (a,b)
				Tp63	Gr1 > Gr4 (b)
				TNF-α	Gr2 > Gr4, Gr3 > Gr5 (a,b)
				wnt5α	Gr2 > Gr4 (b)
				Correlation: p65 and TP63	S(a); negative S (b)
				TP53 and TP63	Negative S(a); S (b)
			Cells' viability	Cells' viability	CT > Gr1 Gr2 = Gr3 = Gr7
			FACS	G2/M cells' percent	Gr2,Gr3 > Gr1
Shellman, 2017	Prospective	FaDu cells exposed to 24 h	ELISA	TGF-β1	Gr1,3,4 > Gr5
(31)	Experimental Controlled	Gr1: cholic acid (CA) Gr2: lithocholic acid (LA) Gr3: deoxycholic acid (DA) Gr4: chenodeoxycholic acid (CDA) Gr5: CT	Cell Titer-Blue assay	MMP9 Cell viability	Gr2 = Gr5 Gr2,3 > Gr5 Gr1,4 = Gr5 Gr1,2,3,4,5 > 95%
Doukas, 2018	Prospective	Human hypopharynx samples	Luciferase assay	NF-κB transcriptional activity	Gr1 > Gr2 (a,b)
(32)	Experimental Controlled	HHPCs, HHK (a,b) Exposed to bile fluid 10-15 min, 3/d, 5d, pH 4.0, 7.0 NF-κB inhibitor: BAY 11-7082 Gr1: BA, pH 4 Gr2: BA+ BAY, pH 4 Gr3: BA, pH 7 Gr4: BA+ BAY, pH 7 Gr5: CT	qPCR	miR-21, -155, and -192 miR-34a, -375, and -451a miR-21/miR-375	Gr3 > Gr4 (a,b) Gr1 > Gr5, Gr2 < Gr1 (a,b) Gr2/Gr1 < Gr4/Gr3 (a,b) Gr3 > Gr5, Gr4 < Gr3 (a,b) Gr1 < Gr5, Gr2 > Gr1 (a,b) Gr2/Gr1 > Gr4/Gr3 (a,b) Gr3 < Gr5, Gr4 > Gr3 (a,b) Gr1 > Gr3, Gr5 (a), Gr1 > Gr5 (b) Gr2 < Gr3, Gr5 (a),Gr2 < Gr5 (b) Gr1 > Gr2 (a,b)
			Immunofluorescence Cell viability assay	STAT3, NF-κB Cell viability	Gr1 > Gr2 (a), Gr1 > Gr3 (a) Gr1 < Gr2, Gr3 > Gr4 (a,b)

TABLE 2 (*Continued*)

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Vageli, 2018	Prospective	Human hypopharynx samples	Immunofluorescence	p-NF-κB, Bcl-2	Gr1 > Gr2 (a)
(33)	Experimental Controlled	HHPCs, HHK (a,b)	WB		Gr2 < Gr4,5,6 (a,b)
		Exposed to bile fluid		Nucleus NF-κB, cytoplasmic p-IκB-α	Gr3 = Gr4 (a)
		10–15 min, 3/d, 5d, pH 4.0, 7.0		NF-κB transcriptional activity	Gr1 > Gr2 (a,b)
		NF-κB inhibitor: BAY 11-7082			Gr3 = Gr4 (a,b)
		Gr1: BA, pH 4	qPCR	Antiapoptotic and oncogenic	Gr1 > Gr3,5,6 (a,b)
		Gr2: BA+ BAY, pH 4			Gr1 < Gr2 (a,b)
		Gr3: BA, pH 7		RELA, STAT3, EGFR, bcl-2, and IL1β	Gr1 < Gr2 (a,b)
		Gr4: BA+ BAY, pH 7		c-REL	Gr1 > Gr2, Gr3 > Gr4 (a)
		Gr5: pH 4		mRNA change ratio	Gr2/Gr1 < > Gr4/Gr3 (a,b)
		Gr6: pH 7		84 genes about NF-κB	Gr2 < Gr1 (72 down, a)
				MAP3K1	Gr2 < Gr1 (a)
				ATF1, EGR1, ELK1, FOS, and STAT1	Gr2 < Gr1 (a)
				BCL10, CARD11	Gr2 < Gr1 (a)
Vageli, 2018	Prospective	Human hypopharynx samples	Immunofluorescence	Nucleus p65	Gr1 > Gr2
(34)	Experimental Controlled	HHPCs	WB	bcl-2	Gr1 > Gr2, Gr3 > Gr4
		Exposed to bile acid	Luciferase assay	NF-κB transcriptional activity	Gr1 > Gr2
		10 min, 2/d, 4d, pH 4.0, 7.0	RT-qPCR	RELA (p65), c-Rel, bcl-2, TNF-α, EGFR, STAT3, WNT5A, ΔNp63, and IL-6	Gr1 > Gr3,5,6
		Gr1: BA, pH 4			Gr1 < Gr2
		Gr2: BA+ curcumin, pH 4		RELA, STAT3, and EGFR	Gr1 < Gr2, Gr3 < Gr4
		Gr3: BA, pH 7		bcl-2	Gr1 < Gr2, Gr3 < Gr4
		Gr4: BA+ curcumin, pH 7		p-STAT3	Gr1 > Gr2
		Gr5: pH 4	PCR array	84 genes about NF-κB	Gr1: 66 up, Gr2: 20 down
		Gr6: pH 7		NFkB2, TNFRSF1A, and TNFRSF10	Gr1 > Gr2
				BIRC2, CARD11, LTA, TICAM1, and TRAF3	Gr1 > Gr2
				CHUK, bcl-3	Gr1 > Gr2
			Cell viability assay	Cell viability	Gr1 > Gr2, Gr3 > Gr4

TABLE 2 (*Continued*)

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Doukas, 2019	Prospective	Human hypopharynx samples	Immunofluorescence assay	p-NF-κB, p-STAT3, and Bcl-2	Gr1 > Gr2 > Gr3
(35)	Experimental	HHPCs	and WB		
	Controlled	Exposed to	Luciferase assay	NF-κB transcriptional activity	Gr1 > Gr2 > Gr3
		bile acid (BA), 7 min, 2/d, 4d	RT-qPCR	bcl-2, RELA, and c-REL	Gr1 > Gr2,Gr3
		Gr1: BAs, pH 4		EGFR, STAT3, TNF-α, and ΔNp63	Gr1 > Gr2,Gr3
		Gr2: BA, pH 5.5		RELA, c-REL, STAT3, and TNF-α	Gr2 > Gr3
		Gr3: BA, pH 7.0		RELA, STAT3, TNF-α, EGFR, and ΔNp63	Gr3 > Gr6
		Gr4: pH 4.0		84 genes about NF-κB	Gr1: 39 up
		Gr5: pH 5.5		EGR1, ELK1, and CARD11	Gr1 > Gr2,Gr3
		Gr6: pH 7.0		RELA, NF-κB2, TNFRSF-10B, -1A, -10, and -14	Gr1 > Gr6
				BIRC2, IRF1, LTA, TRAF3, -6, and IL1A	Gr1 > Gr6
				IKBKB, IKBE, BCL-3, BIRC3, and TLR-3, -4, -6	Gr1 > Gr6
Sasaki, 2020	Prospective	Human hypopharynx/larynx samples	Immunofluorescence	p-IKB-α	Gr1 > Gr2,3 (a,b)
(36)	Experimental	FaDu, UMSCC11A(a,b) exposed to	WB		Gr2 > Gr5, Gr3 > Gr6 (a)
	Controlled	bile acid (BA), pH 4.0, 5.5, 7.0		bcl-2	Gr1,2 > Gr3, Gr3 > Gr6 (a)
		7 min, 3/d, 4d		Correlation: p-IKB-α and total p65	S (a)
		Gr1: BAs, pH 4		Correlation: p-IKB-α and p-p65	S (b)
		Gr2: BA, pH 5.5		Correlation: p-IKB-α and Bcl-2	S (a,b)
		Gr3: BA, pH 7.0	qPCR	NF-κB transcriptional activity	Gr1 > Gr2,3 (a)
		Gr4: pH 4.0			Gr1,2 > Gr3, Gr3 > Gr6 (a)
		Gr5: pH 5.5		TNF-α, IL-6	Gr1 > Gr2,3 (a,b)
		Gr6: pH 7.0		BCL-2, RELA, STAT3, and WNT5A	Gr1 > Gr2,3 (a,b)
				IL1β, c-REL, and p63	Gr1 > Gr2,3 (a)
				IL-6	Gr2 > Gr3,Gr5 (a,b)
				TNF-α	Gr2 > Gr5 (a), Gr3 > Gr6 (b)
				EGFR	Gr2 > Gr5 (b)
				IL-6, BCL-2	Gr3 > Gr2,Gr6 (b)
				c-Rel mRNA	Gr3 > Gr6 (b)
			Cell viability assay	Cell viability	Gr1 > Gr2,3 (a,b)
					Gr1 > Gr4, Gr2 > Gr5, Gr3 > Gr6

TABLE 2 (*Continued*)

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
Doukas, 2021	Prospective	Human hypopharynx samples	Cell viability assay	Cell viability	Gr2,3,4 > Gr1
(37)	Experimental Controlled	Exposed to pig pepsin, 15 min, 1/d, 4-5d	Immunofluorescence WB and ELISA	Pepsin in cells p-EGFR, Bcl-2	Gr3 > Gr1,Gr2 Gr2,3 > Gr1,4, Gr3 > Gr2
		Gr1: pepsin, pH 5		p-EGFR	Gr1 = Gr4
		Gr2: pepsin, pH 6		Bcl-2	Gr1,Gr2 > Gr4
		Gr3: pepsin, pH 7		STAT3, NF-κB (p65)	Gr3 > Gr2 > Gr1 > Gr4
		Gr4: CT, pH 7		EGFR, IL-6, IL1β, and RELA	Gr3 > Gr2 > Gr1
				mTOR, AKT1, TNF-α, STAT3, and BCL-2	Gr3 > Gr2 > Gr1
				TNF-α, Il6	Gr1 > Gr4
				PTGS2, PKI3CA	Gr1 = Gr2 = Gr3 = Gr4
				Correlation: p-EGFR and p-NF-κB	S
				Correlation: p-EGFR and pepsin A	S
Samuels, 2021	Prospective	Human larynx samples	RNA sequencing and	(FC > 1.3, FDR < 0.05)	397
(52)	Experimental Controlled	Healthy true vocal cord cell Exposed to pig pepsin, 1 h, pH 7.0		FDR < 1×10^{-6}	171
				Chemo dependency	35.00%
				Hematologic diseases	16.00%
		Gr1: pepsin		Cancer development	24.00%
		Gr2: CT		Metabolism	40.00%
			Pathway analysis	Histone methyltransferases	S
				KRAS,TNFA/NF-KB	S
				DNA repair, proliferation, and oncogenic transformation	S
Doukas, 2023	Prospective	Human hypopharynx samples	Immunofluorescence	DNA, oxidative stress-related damage	Gr1 > Gr7
(38)	Experimental Controlled	Exposed to BA, pH 4		PARP-1, p-NF-κB	Gr1 > Gr7
				DNA damage, PARP-1	Gr1 > Gr5
		Gr1: BA		DNA damage, p-NF-κB	Gr1 = Gr3 > Gr5
		Gr2: BA + si-RNA		PARP-1	Gr1 > Gr3
		Gr3: BA +AGO		Oxidative damage	Gr1,Gr3 > Gr5
		Gr4: BA +si-PARP-1	Cell viability assay	Cell viability	Gr1 = Gr3,4
		Gr5: BA + BAY			Gr1 > Gr5,6
		Gr6: BA + si-NF-κB	Luciferase assay	NF-κB transcriptional activity	Gr1 = Gr3,4
		Gr7: acid			Gr1 > Gr5,6
		Gr8: BA + si-RNA	ELISA	PARP-1, p-NF-κB, and Bcl-2	Gr1 > Gr5,6, Gr1 > Gr7
				PARP-1	Gr1 > Gr3,4

TABLE 2 (*Continued*)

References	Design	Sample/ Patients' Characteristics	Analysis	Outcomes	Results
				p-NF-κB	Gr3,4 > Gr1
				Bcl-2	Gr1 = Gr3,4
			qPCR	PARP-1 mRNA	Gr1 > Gr3,4
				NF-κB mRNA	Gr1 = Gr3,4
				PARP-1,, STAT3, EGFR, RELA, BCL-2, IL-6, IL1β, TNFα, PTGS2, and WNT5a	Gr1 > Gr5,6
				STAT3, TNFα, RELA, IL1β, and EGFR	Gr1 > Gr4
				Bcl-2, PTGS, WNT5a, and IL-6	Gr1 < Gr4
Yu, 2024 (39)	Prospective Experimental	Laryngeal carcinoma cells TU212, TU 686 (a,b) exposed to	WB	Glut-1	Gr1 > Gr3,5, Gr4 > Gr3
			Radio immunoassay	18F-FDG uptake, lactate	Gr1,4 > Gr5
	Controlled	acidic pepsin,15 min/d, 3/d, 5d, pH 3			Gr1 > Gr2, Gr1,Gr5 > Gr3
		Gr1: pepsin	CCK-8	Cell viability	Gr4 > Gr3,5, Gr1 > Gr2,3
		Gr2: pepsin + 2-DG	Immunofluorescence	Ki67	Gr4 > Gr5 > Gr3, Gr1 > Gr2,3
		Gr3: pepsin + si-Glut-1	Transwell assay	Migration and invasion	Gr4 > Gr5 > Gr3, Gr1 > Gr2,3
		Gr4: pepsin + si-RNA/Gr5: pH 3	Clonal formation assay	Colony number	Gr4 > Gr5 > Gr3, Gr1 > Gr2,3

Abbreviations: ATF1, activating transcription factor 1; BA, bile acid; BCL10, B-cell lymphoma/leukemia 10; BCL2A1, BCL-2-related protein A1; c-Myc, myelocytomatosis oncogene; CARD11, caspase recruitment domain-containing protein 11; CHUK, conserved helix-loop-helix ubiquitous kinase; CT, control group; EGR1, early growth response 1; ELK1, ETS-like transcription factor 1; FaDu, human hypopharyngeal squamous cell carcinoma cell line (FaDu); FDR, false discovery rate; FOS, fos proto-oncogene; Gr, group; Hep-2, human laryngeal epithelial carcinoma cell line; HHK, human hypopharyngeal keratinocytes; HHPCs, human hypopharyngeal primary cells; HPSCC, hypopharyngeal squamous cell carcinoma; IKBKB, inhibitor of nuclear factor kappa B kinase subunit beta; IRF1, interferon regulatory factor 1; LTA, lymphotoxin alpha; m, minute; MAP3K1, mitogen-activated protein kinase kinase kinase 1; N, number; N.P, not provided; NFκB2, nuclear factor kappa B subunit 2; p21, cyclin-dependent kinase inhibitor 1A; PARP-1, poly(ADP-ribose) polymerase 1; Ras protein, rat sarcoma viral oncogene homolog protein; SNU-1041, human hypopharyngeal squamous cell carcinoma cell line SNU-1041; STAT1, signal transducer and activator of transcription 1; TFs, transcription factors; TICAM1, TIR domain-containing adapter molecule 1; TLR-3, Toll-like receptor 3; TLR-4, Toll-like receptor 4; TLR-6, Toll-like receptor 6; TNFRSF10, TNF receptor superfamily member 10; TNFRSF1A, TNF receptor superfamily member 1A; TRAF3, TNF receptor-associated factor 3; TRAF6, TNF receptor-associated factor 6; TU212, human head and neck squamous cell carcinoma cell line TU212; UMSCC-11B, University of Michigan squamous cell carcinoma cell line 11B; w, week.

TABLE 3.
Animal Model Studies

References	Design	Sample/Patients' Characteristics	Analysis	Outcomes	Results
Johnston, 2006	Prospective	Porcine laryngeal samples *in vitro*	WB	Hsp70	Gr1 = Gr2
(18)	Experimental Controlled	Exposed to 1 mg/mL pepsin pH 2,4,7.4, 20 min, 3 h Gr1: Treat group, Gr2: CT		Sep53, Sep70	Gr2 > Gr1
Ling, 2007	Prospective	Rats' larynx and esophagus samples	IHC	Larynx	
(40)	Experimental Controlled	Gr1: larynx, N = 16	Ki67	Inflammatory cells' infiltration	Gr1 > Gr3 (a,b,c)
		Gr2: esophagus, N = 16		Laryngitis and small glands' proliferation	Gr1 > Gr3 (b,c)
		Gr3: sham-operated, N = 5		Basal cell and ciliated cells' hyperplasia	Gr1 > Gr3 (c)
		a,b,c = 10w, 20w, and 30w			
Del Negro, 2008	Prospective	Rats' pharyngeal mucosa samples		Dysplasia, neoplasia, and invasive cancer	NS in all groups
(41)	Experimental	(N = 82), exposed to hydrochloric acid		Mucosal ulceration	NS in all groups
	Controlled	Sodium nitrate (SN), pepsin		Inflammatory histological changes	S (varies in all groups)
		N = 12 each experimental group for 6 M		Lymphocytes, mast cell infiltrate	S in all groups
		Gr1: HCL, Gr3: HCL+pepsin 2 /w		Mast cells	Gr5,6 > Gr1,2,3,4 > Gr7
		Gr2: HCL, Gr4: HCL+pepsin 3 /w			
		Gr5,Gr6: HCL+SN 2/w, 3/w			
		Gr7: N = 10 filtered water 2/w			
Sasaki, 2016	Prospective	Murine larynx samples	Histological analysis	Hyperplasia	Gr4 > Gr7
(42)	Experimental	N = 35, 5 each group		Dysplasia	Gr2,4 > Gr7
	Controlled	Exposed to		Thickness	Gr1,2,3 > Gr5,7
		bile salts (BA), 150-200 μL, 2/d, 45d	IHC	p-NF-κB	Gr1,2,3,4 > Gr6,7
		Gr1: BA, pH 3		p-NF-κB, ΔNp63	Gr1 > Gr5,7, Gr2,4 > Gr7
		Gr2: BA, pH 7.0		Ki67	Gr1 > Gr5,7, Gr2,3,4 > Gr5
		Gr3: DCA, pH 7.0		E-cadherin	Gr1,2 > Gr7
		Gr4: CDCA, pH 7.0		CK14, β-catenin	Gr1 > Gr5,7
		Gr5: acid alone, pH 1.5		β-catenin	Gr4 > Gr7
		Gr6: glucose (33 mM), pH 7.0		p-STAT3	Gr1 > Gr7
		Gr7: CT untreated control		Correlation	
				NF-κB and ΔNp63, p-STAT3, and β-catenin	S
				ΔNp63 and CK14, p-STAT3	S
				E-cadherin and β-catenin	Negative S

TABLE 3 (*Continued*)

References	Design	Sample/Patients' Characteristics	Analysis	Outcomes	Results
			RT-qPCR	miR-21, -155, -192	Gr1 > Gr7
				miR-155	Gr2,3,4 < Gr7
				miR-375	Gr7 > Gr1,4, Gr2,3 > Gr7
				miR-34a, -451a	Gr7 > Gr1
				miRNA disorders	Gr1 > Gr2,3,4
				Correlation: miRNA disorders and p-NF-κB	S
				Correlation: miR-21 and miR-155 and miR-192	S
Vageli, 2016 (43)	Prospective Experimental Controlled	Murine hypopharynx samples N = 40, 5 each group Exposed to bile salts (BA), 0.15 mL, 2/d, 45d	Histological analysis	Hyperplasia	Gr1,2,3,4 > Gr7
				Dysplasia	Gr1,3,4 > Gr7
				Thickness	Gr1,2,3,4 > Gr7
				Histological change	Gr5 = Gr6 = Gr8
		Gr1: BA, pH 3	Immunofluorescence	p-NF-κB, Bcl-2	Gr1 > Gr5, Gr1 > Gr1,Gr8
		Gr2: BA, pH 7.0	WB	Ki67, CK14	Gr1,2,3,4 > Gr5,7
		Gr3: DCA, pH 7.0		ΔNp63	Gr1,3,4,5 > Gr2,7
		Gr4: CDCA, pH 7.0		Correlation	
		Gr5: acid alone, pH 1.5		IκB-α and Bcl-2	S
		Gr6: glucose (33 mM), pH 7.0		p-NF-κB and Bcl-2	S
		Gr7: untreated control		Nucleus p65	Gr1,2,3,4 > Gr5 > Gr6,7,8
		Gr8: saline, pH 7.0		p-NF-κB, Ki67, ΔNp63, p-STAT3, CK14, and β-catenin	Gr1,2,3,4 > Gr5 > Gr7
		Hypopharyngeal tissue		E-cadherin	Gr1,2,3,4 < Gr7
				Correlation	
				p-NF-κB and Ki67, CK14, p-STAT3, and β-catenin	S
				RELA (p65), bcl-2, EGFR, WNT5A, TNF-α, and STAT3	Gr1,3,4 > Gr7
				Correlation	
			RT-qPCR	RELA (p65) and bcl-2, TNF-α, and WNT5A	S
Doukas, 2019 (44)	Prospective Experimental Controlled	Murine hypopharyngeal primary cells Exposed to bile acid (BA), 7 min, 2/d, 3d	immunofluorescence assay	p-p65, bcl-2	Gr1 > Gr2,Gr3
			WB		Gr3 > Gr2 (15 min) Gr2 = Gr3 (5,10 min)
		NF-κB inhibitor: BAY 11-7082, 15 min	Luciferase assay	NF-κB transcriptional activity	Gr1 > Gr4,Gr1 > Gr2,3
		Gr1: BA, pH 4		EGFR, bcl-2, and IL-6,	Gr1 > Gr2,3, Gr1 > Gr4
		Gr2: pre BAY + BA, pH 7		RELA, STAT3, and WNT5A	Gr1 > Gr3 > Gr2

TABLE 3 (*Continued*)

References	Design	Sample/Patients' Characteristics	Analysis	Outcomes	Results
		Gr3: BA+ post BAY, pH 7		TNF-α	Gr1 = Gr2 > Gr3
		Gr4: DMSO, pH 7	RT-qPCR	miR-21, -155, -192	Gr1 > Gr2,Gr3
		Records: 5, 10, and 15 min		miR-21	Gr3 > Gr2
				miR-21/375	Gr1 > Gr2,Gr3
				miR-34a, -375, -451a	Gr1 > Gr2,Gr3
				miR-34a, -375	Gr2 > Gr3
				miR-451a	Gr3 > Gr2
				Correlation: RELA and miR-155, -192	S in Gr2,Gr3
				Correlation: RELA and miR-34a	Negative S in Gr2,Gr3
Doukas, 2020	Prospective	Mus musculus hypopharynx samples	Immunofluorescence assay	NF-κB activation, Ki67	Gr1 > Gr2,3,4
(45)	Experimental Controlled	N = 48, 8 for each group	WB		Gr1 > Gr5,Gr6 (S)
		Exposed to	qPCR	Rela, Bcl-2, EGFR, Stat3, Wnt5a, TNF, Il6, and Ptgs2	Gr1 > Gr2,3,4
		bile acid (BA), 3/d, 10d		RELA, Wnt5a, Bcl-2, and Ptgs2	Gr2 = Gr4 > Gr3
		Curcumin (Cur), 2/d, 10d		EGFR	Gr4 > Gr2,3
		Gr1: BA, pH 3		AKT1	Gr1 > Gr4 > Gr3, Gr1 = Gr2
		Gr2: pre Cur + BA, pH 3		mTOR	Gr2,4 > Gr1
		Gr3: co-Cur + BA, pH 3		Rela and EGFR, Wnt5a, and Ptgs2	S
		Gr4: BA + post Cur, pH 3		Correlation: Stat3 and IL-6/Egfr and Ptgs	S
		Gr5: DMSO, pH 7/Gr6: CT		TNF and Akt1	S
Sasaki, 2020	Prospective	Mus musculus hypopharynx samples	Histological Analysis	Hyperplasia, dysplasia	Gr1 > Gr3 (a)
(46)	Experimental Controlled	N = 48, 8 for each group		Microinvasion	Gr1 > Gr3 (a)
		Exposed to		Malignant invasion	Gr1 > Gr3 (b)
		bile acid (BA), 2/d, 45, 100d	IHC	Ki67, CK14	Gr1 > Gr2,3 (a,b), Gr1 (b > a)
		45,100d (a,b)		γH2AX	Gr1 > Gr2,3 (a,b), Gr1 (b > a)
		Gr1: BA, pH 3		DNA/RNA damage	Gr1 > Gr2,3 (a,b), Gr1 (b > a)
		Gr2: acid, pH 3		E-cadherin	Gr2,3 > Gr1 (a,b), Gr1 (a > b)
		Gr3: saline, pH 7		p-NF-κB, P53	Gr1 > Gr2,3 (a,b), Gr1(b > a)
			PCR array	TNF, IL-6, EGFR, Bcl-2, Stat3, Wnt5a, and Rela	Gr1 > Gr2,3 (a,b), Gr1 (b > a)
				miR-21, -155, -192	Gr1 > Gr2,3 (b)
				miR-155, -192	Ge1 (b > a)
				miR-155	Gr2 > Gr3
				miR-34a, -375, -451a	Gr1 < Gr2,3 (b)
				miR-375, -451a	Gr1 (a > b)

TABLE 3 (*Continued*)

References	Design	Sample/Patients' Characteristics	Analysis	Outcomes	Results
				miR-34a	Gr1 (b > a)
Vageli, 2020	Prospective	Mus musculus hypopharynx samples	IHC	Nucleus p65	Gr1 > Gr4,Gr5
(47)	Experimental Controlled	N = 40, 8 for each group		and Ki67	Gr1 > Gr2,Gr3
		Exposed to		Nucleus p65	Gr3 > Gr2
		bile acid (BA), 2/d, 10d		Rela, Stat3, EGFR, Bcl-2, Wnt5a, TNF, Il6, and Ptgs2	Gr1 > Gr4,Gr5
		BAY 11-7082			Gr1 > Gr2,Gr3/Gr3 > Gr2
		Gr1: BA, pH 3	qPCR	miR-192, miR-21, and miR-155	Gr1 > Gr4,Gr5
		Gr2: pre BAY + BA, pH 3			Gr1 > Gr2,Gr3
		Gr3: BA + post BAY, pH 3		miR-375, miR-34a, miR-451a, miR-504, and miR-99a	Gr4,Gr5 > Gr1
		Gr4: DMSO, pH 7			Gr2,Gr3 > Gr1
		Gr5: untreated		miR-21, miR-155	Gr2 > Gr1,Gr3
				miR-99a	Gr1,Gr3 > Gr2
				Correlation: miR-21 and miR-155	Positive S
				Correlation: miR-34a and miR-451a	Positive S
				Correlation: miR-21 and miR-99a	Negative S
				Correlation: miR-155 and miR-99a	Negative S
Sasaki, 2021	Prospective	Mus musculus hypopharynx samples	Histology	Dysplasia, microinvasion	Gr1 > Gr2
(48)	Experimental Controlled	N = 32, 8 for each group	IHC	Malignant invasion	Gr1,Gr2 > Gr4
		Exposed to		E-cadherin	Gr4 > Gr1,Gr2
		bile acid (BA), 2/d, 15w		Ki67, CK14	Gr1,Gr2 > Gr4
		Gr1: BA (with DCA), pH 5.5		DNA damage, γH2Ax	Gr1,Gr2 > Gr4
		Gr2: BA (without DCA), pH 5.5		NF-κB activation, P53	Gr1,Gr2 > Gr4
		Gr3: saline, pH 5.5	qPCR	Bcl-2, Il6, TNF, EGFR, Wnt5a, Rela, and Stat3	Gr1 > Gr2,3,4
		Gr4: saline, pH 7		TNF, bcl-2	Gr1,Gr2 > Gr4
				EGFR, IL-6, Wnt5a, and Rela	Gr1 > Gr4
				miR-21, -155	Gr1,2 > Gr4
				miR-375, -451a	Gr4 > Gr1,2/Gr4 > Gr3
				miR-451a	Gr4 > Gr1
				Correlation: miR-451a and Thf	Negative S
				Correlation: miR-155 and Stat3	Positive S

TABLE 3 (*Continued*)

References	Design	Sample/Patients' Characteristics	Analysis	Outcomes	Results
Chen, 2022	Prospective	Rat larynx samples	Flow cytometry	Cell viability	Gr3 > Gr1,Gr2 (a,b, a > b)
(49)	Experimental	Larynx cancer cell (AMC-HN-8)(a)	Apoptosis and	Apoptosis	Gr1,Gr2 > Gr3 (a,b, a < b)
	Controlled	Normal larynx cell (b)	autophagy	Autophagy	Gr1,Gr2 > Gr3 (a,b, a < b)
		Exposed to	Detection kits	ERS-related protein	Gr1,Gr2 > Gr3 (a,b, a < b)
		pepsin (mg/mL), pH 7	IHC	HSP70 expression	Gr1,Gr2 > Gr3 (a,b, a < b)
		Gr1: 0.1 mg/mL pepsin	WB	Cell viability	G4 > G5 (a,b)
		Gr2: 1 mg/mL pepsin		Apoptosis	Gr5 > G4 (a,b)
		Gr3: CT		Autophagy	Gr5 > G4 (a,b)
		G4: shRNA-NC+pepsin		HSP70, GRP78, cleaved caspase-4, IREα, and XBP-1S	G4 > G5 (b)
		G5: shRNA-HSP70+pepsin		CHOP	G4 = G5 (b)
		G6: CT	IHC	HSP70, GRP78	G4 > G5 (a)
		20 BLAB/c nude mice	TUNEL	Apoptosis	Gr2,3 > Gr4, Gr4 > Gr1
		injected with AMC-HN-8 cells, 28d	staining	Autophagy	Gr1,2 > Gr4, Gr4 > Gr3
		Gr1: +ML346		HSP70	Gr1 > Gr4, Gr4 > Gr2,3
		Gr2: +VER-155008		CHOP	Gr4 > Gr1, Gr4 > Gr2,3
		Gr3: +4-PBA		GRP78	Gr1 = Gr4, Gr2 > Gr4, Gr4 > Gr3
		Gr4: CT		IREα, XBP-1S, and procaspase-12	Gr4 > Gr1,3/Gr2 > Gr4
Vageli, 2022	Prospective	Mus musculus hypopharynx samples	Histological analysis	Severe dysplasia	Gr2,3 > Gr4
(50)	Experimental	N = 40, 8 each group exposed to	IHC	In situ carcinoma	Gr2 > Gr4
	Controlled	TCS: components of tobacco smoke		Invasive malignancy	Gr1 > Gr4
		NNK 0.2 mol/L, NDEA 0.004 mol/L		Nucleus p-NF-κB	Gr1 > Gr2 > Gr4
		Add in water for only drink, 5d/w	qPCR	NF-κB oncogenic gene	Gr1 > Gr2 > Gr4
		Bile acid, DCA, 2/d, 5d/w		Il6, TNF, Stat3, EGFR	Gr2 > Gr4
		Gr1: TSC+DCA		and Wnt5a, Rela, mTOR, and Bcl-2	Gr1 > Gr2,3 > Gr4
		Gr2: TSC - Gr3: DCA		Correlation: Il6, TNF, Stat3, EGFR	
		Gr4: saline - Gr5: untreated		and Wnt5a, Rela, mTOR, and Bcl-2	Positive S

Abbreviations: 18F-FDG, fluorodeoxyglucose F-18; CHOP, C/EBP homologous protein; CK14, cytokeratin 14; GRP78, glucose-regulated protein 78; IRE1α, inositol-requiring enzyme 1 alpha; M, month; XBP-1S, spliced X-box-binding protein 1; γH2AX, phosphorylated histone H2AX.

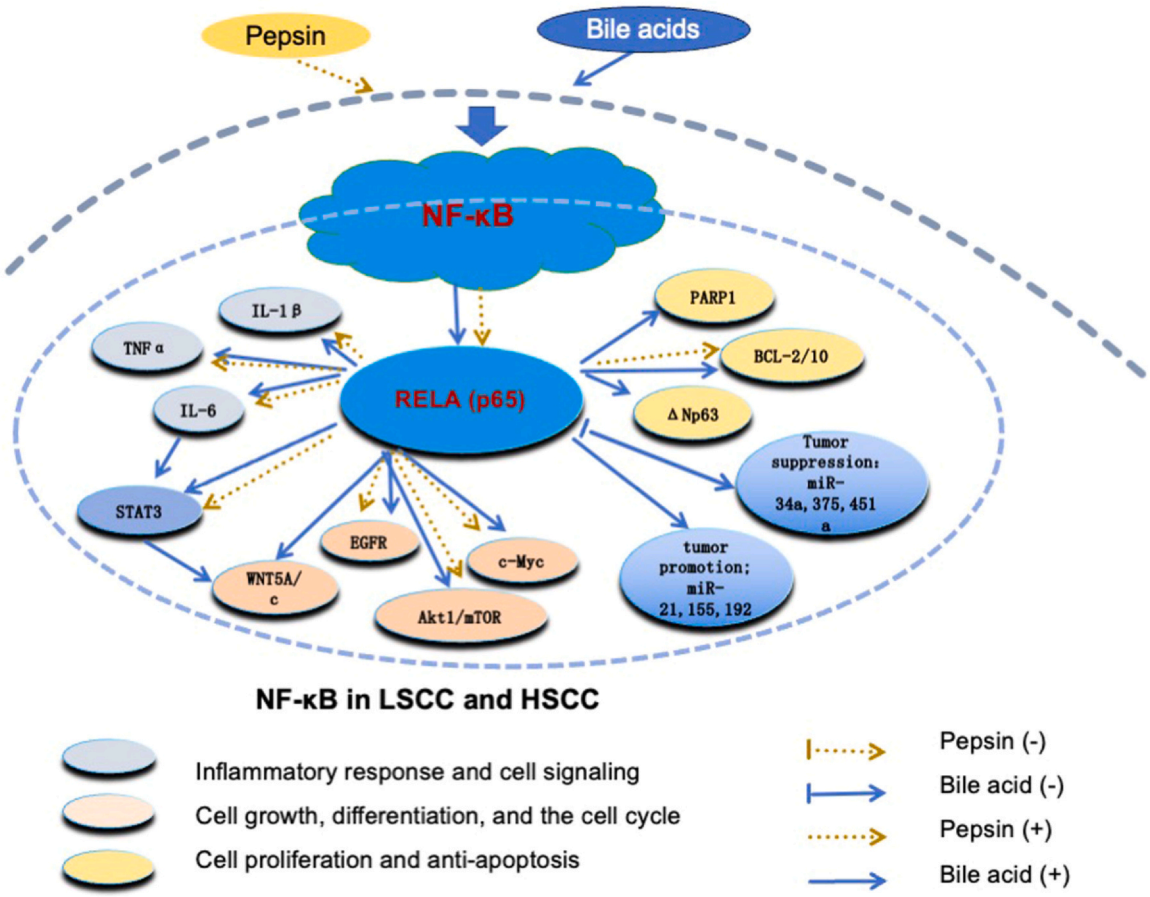

FIGURE 2. Summary of cell activation and pathway in reflux disease. The mechanism of NF-κB pathway in laryngeal and hypopharyngeal squamous cell carcinomas under the influence of laryngopharyngeal reflux disease content. As shown in the figure, pepsin activates the NF-κB pathway, leading to the release of inflammatory mediators, enhanced resistance to apoptosis, and affecting cell proliferation and differentiation. Bile acids also participate in the above processes and influence epigenetic changes through this pathway.

this study, there was no significant association between the expression of H^+/K^+-ATPase and the prognosis outcomes. In 2019, Sasaki et al observed an overactivation of NF-κB pathway, accompanied by significant overexpression of RELA(p65), EGFR, STAT3, BCL-2, c-REL, ΔNp63, WNT5A, IL-6, and IL-1B, which are involved in tumor initiation, progression, immunosuppressive properties, and metastasis (Table 4). Moreover, the authors demonstrated significantly higher mRNA levels of all genes, particularly RELA(p65), IL-6, EGFR, and TNF-α compared with bile acid-negative tumor samples.[24] The roles of these molecules in carcinogenesis are summarized in Table 4. Johnston et al reported decreased Sep70, Sep53, and Hsp70 expression in laryngeal tumors exposed to reflux content (eg, pepsin) compared with healthy tissues, which contributed to tumor progression.[18] Tan et al investigated the relationship between pepsin and LSCC using LSCC samples and two human LSCC-derived cell lines (Hep-2 and Tu212). In this study, they demonstrated upregulation of IL-8, β-catenin, and Vimentin expression, alongside downregulated E-cadherin expression in pepsin-positive laryngeal tumor tissues, which correlated with pepsin expression intensity.[22]

Human cell line studies

All studies focused on pepsin or bile acid effects on human-derived laryngeal or pharyngeal cell lines. Bile acids were investigated in 10 studies.[23,26,29,31–36,38] Six studies examined the impact of bile acid mixtures on the NF-κB pathway under various conditions.[29,33–36,38] The authors observed comparable effects to pepsin exposure, which consisted of an enhancement of cell viability and upregulation of the expression of numerous molecules involved in tumor initiation, progression, proliferation, and invasiveness, including the NF-kB pathway molecules (BCL-2, RELA(p65), c-REL, STAT3, ΔNp63, EGFR, WNT5A, and TNF-α).[29,33–36,38]

Regarding the importance of NF-kB pathway, Vageli et al treated hypopharyngeal cells with curcumin, leading to a successful inhibition of the acidic bile-induced NF-kB signaling pathway, and an overexpression of NF-kB transcriptional factors, c-REL, RELA(p65), antiapoptotic

TABLE 4.
Summary of Bile Acid and Pepsin Effects in Laryngeal and Hypopharyngeal Carcinogenesis

Sample	LPRD Content	Expression	Molecules	Potential Literature-Based Roles in Laryngopharyngeal Squamous Cell Carcinomas
Human	Bile acids	Overexpression	RELA (p65)	Subunit of the NF-κB transcription factor complex regulating genes involved in inflammation, cell survival, and proliferation.
Tissue				*Constitutive activation of RELA/p65 promotes cancer cell survival, angiogenesis, and metastasis.*
			EGFR	Trigger of multiple signaling cascades promoting cell proliferation, survival, and migration.
			STAT3	Transcription factor promoting expression of genes involved in cell cycle progression, apoptosis resistance, and angiogenesis.
			BCL-2	Antiapoptotic protein that prevents programmed cell death by inhibiting cytochrome-C release from mitochondria.
				Overexpression of BCL-2 contributes to cancer cell survival and resistance to therapy.
			c-REL	NF-κB family member of transcription factors regulating genes involved in immune response, inflammation, and cell survival.
			ΔNp63	Dominant-negative regulator of p53 family members.
				Overexpression is associated with proliferation, survival, and stemness properties.
			WNT5A	Ligand in the noncanonical Wnt signaling pathway involved in cell migration, differentiation, and polarity.
				WNT5A exhibits context-dependent tumor-promoting or tumor-suppressing functions.
			IL-6	Pro-inflammatory cytokine that activates JAK/STAT signaling, particularly STAT3.
				Elevated IL-6 contributes to inflammation-associated carcinogenesis, tumor growth, angiogenesis, and metastasis.
			IL-1B	Pro-inflammatory cytokine activating NF-κB signaling and promoting expression of genes involved in inflammation, cell proliferation, and survival.
				Chronic IL-1B-mediated inflammation contributes to tumor initiation, progression, and immunosuppression tumor microenvironment.
	Pepsin	Downregulation	Sep70	Selenium-binding protein involved in detoxification processes, oxidative stress protection, and cell growth regulation.
				A decreased expression may be associated with tumor progression.
			Sep53	Selenium-binding protein involved in selenium metabolism and cellular redox regulation.
				Dysregulation of Sep53 may contribute to carcinogenesis through impaired antioxidant defense mechanisms.
			Hsp70	Chaperone that assists in protein folding, transport, and degradation.
				The downregulation reduces cellular protective mechanisms against acid and enzyme injury.
			E-cadherin	Calcium-dependent cell adhesion molecule predominantly expressed in epithelial tissues.
				Downregulation is associated with tumor progression through an increase of local invasion and distant metastasis.
	Pepsin	Upregulation	IL-8	Pro-inflammatory chemokine that promotes angiogenesis, tumor-related inflammation, and epithelial-mesenchymal transition.
				IL-8 may enhance invasiveness and the metastatic capability.
			β-catenin	Protein that is the central effector of canonical Wnt signaling.
				Induce transcriptional activation of promoting cell proliferation genes, promote metastatic potential.
			Vimentin	A filament protein expressed by mesenchymal cells, activating cellular migration and invasion, and metastatic potential.

TABLE 4 (*Continued*)

Sample	LPRD Content	Expression	Molecules	Potential Literature-Based Roles in Laryngopharyngeal Squamous Cell Carcinomas
Cell lines	Pepsin	Dysregulation	MALAT1	Long noncoding RNA promoting cancer cell proliferation, migration, and metastatic dissemination.
			Keratin-82	Type II keratin protein contributing to altered cellular structure and mechanical properties.
	Bile acids	Upregulation	COX-2	Enzyme of the prostaglandin biosynthesis involved in the tumor cell proliferation, inhibition of apoptosis, and angiogenesis.
			MMP9	Metallomatrix protease facilitating tumor expression and metastasis through the matrix degradation.
	Bile acids	Overexpression	PARP-1	DNA damage sensor and mediator of base excision repair, maintaining genomic stability by detecting single-strand DNA breaks and facilitating repair mechanisms.
Animals	Pepsin	Overexpression (porcine)	Sep70	cf supra
			Sep53	cf supra
		No change	Hsp70	cf supra
		Overexpression (rat)	Hsp70	cf supra
	Bile acids	Upregulation (mice)	Ki67	Nuclear protein associated with cellular proliferation, expressed during all active phases of the cell cycle (G1, S, G2, and M). *Elevated Ki67 is associated with increased tumor aggressiveness, higher grade, and poorer prognosis.*
			CK14	Type I keratin expressed in basal epithelial cells that form intermediate filaments and maintain structural integrity.
			ΔNp63	cf supra
			STAT3	cf supra
			miRNA-21	Oncogenic microRNA that targets multiple tumor suppressor genes, including PTEN, PDCD4, and TPM1.
			miR-155	Multifunctional microRNA involved in inflammation, immunity, and oncogenesis that regulates numerous targets, including SHIP1, C/EBPβ, and SOCS1.
			miR-192	p53-responsive microRNA that regulates cell cycle progression and epithelial characteristics. *miRNA-21, -155, and -192 are involved in cell proliferation, invasion, metastasis, and therapy resistance when upregulated.*
		Downregulation (mice)	miR-34a	Tumor suppressor microRNA regulated by p53- targeting genes involved in cell cycle, apoptosis, and stemness (BCL-2, SIRT1, and CD44).
			miR-375	Epithelial-specific microRNA that regulates cellular differentiation and insulin secretion in normal tissues.
			miR-451a	Tumor suppressor microRNA that regulates metabolic adaptation, drug resistance, and cell proliferation. *miR-34a, -375, and -451a downregulation reduces cancer cell apoptosis, promoting proliferation, invasion, migration, and therapy resistance.*

Abbreviations: BCL-2, B-cell lymphoma 2; β-catenin, beta-catenin; C/EBPβ, CCAAT/enhancer-binding protein beta; CD44, cluster of differentiation 44; CK14, cytokeratin 14; COX-2, cyclooxygenase-2; c-REL, proto-oncogene c-Rel; ΔNp63, delta Np63 (N-terminally truncated p63); E-cadherin, epithelial cadherin; EGFR, epidermal growth factor receptor; Hsp70, heat shock protein 70; IL-1B, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; JAK, Janus kinase; Ki67, marker of proliferation Ki67; LPRD, laryngopharyngeal reflux disease; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR-155, microRNA-155; miR-192, microRNA-192; miR-34a, microRNA-34a; miR-375, microRNA-375; miR-451a, microRNA-451a; miRNA-21, microRNA-21; MMP9, matrix metalloproteinase-9; PARP-1, poly(ADP-ribose) polymerase 1; PDCD4, programmed cell death protein 4; PTEN, phosphatase and tensin homolog; RELA (p65), v-rel avian reticuloendotheliosis viral oncogene homolog A (p65 subunit); Sep53, stress-exposed protein 53; Sep70, stress-exposed protein 70; SHIP1, SH2-containing inositol phosphatase 1; SIRT1, sirtuin 1; SOCS1, suppressor of cytokine signaling 1; STAT3, signal transducer and activator of transcription 3; TPM1, tropomyosin 1; Vimentin, intermediate filament protein; WNT5A, Wnt family member 5A.

BCL-2, oncogenic TNF-α, EGFR, STAT3, WNT5A, ΔNp63, and cancer-related IL-6. The effect of curcumin being effective for reducing bile-induced bcl-2 overexpression at both acidic and neutral pH.[34] The role of pH was strengthened by Doukas et al, who found that repetitive applications of conjugated primary bile acids with unconjugated secondary bile acid, (deoxycholic acid) on human hypopharyngeal primary cells revealed that strongly acidic pH (4.0) optimally enhanced the tumorigenic effect of bile, by inducing activation of NF-κB, STAT3 nuclear translocation and bcl-2 overexpression, and significant overexpression of the oncogenic mRNA phenotype, compared with weakly acidic pH (5.5) or neutral pH (7.0).[35] Sasaki et al exposed FaDu and UMSCC11A cell lines to bile acids at variable pH points, and demonstrated that bile acids at pH 4.0 potentiated the activation of NF-kB and its related mRNA phenotype, and activated the IL-6, TNF-α, and BCL-2 gene expression. In addition, the authors observed an enhanced transcriptional activity of EGFR, RELA, STAT3, and WNT5A and higher survival rates were observed in HSCC cells exposed to acidic bile compared with those exposed to bile at weakly acidic or neutral pH.[23] In 2023, Doukas et al showed that acidic bile induces PARP-1 overexpression in hypopharyngeal cells. While NF-κB inhibition prevented DNA damage, PARP-1 overexpression, and antiapoptotic phenotypes, PARP-1 inhibition failed to reduce DNA damage, NF-κB activation, or cell survival, suggesting NF-κB as a superior therapeutic target against acidic bile-induced carcinogenesis.[38] Beyond NF-κB activation, the same team reported upregulation of oncogenic miRNAs (miR-21, -155, and -192) and STAT3, alongside downregulation of tumor suppressor miRNAs (miR-34a, -375, and -451a).[32] Concerning the types of bile acids, Shellman et al found that cholic acid, deoxycholic acid, and chenodeoxycholic acid, specifically, promoted TGF-β1 expression, while lithocholic acid and deoxycholic acid promoted matrix metalloproteinase-9 expression.[31] In 2003, Sung et al observed that chenodeoxycholate, which is one of the bile acid components, was found to induce cyclooxygenase-2 (COX-2) expression in human pharyngeal cells, COX-2 being involved in the tumorigenesis of esophageal and pharyngeal squamous cell carcinoma.[26]

Seven studies investigated pepsin effects at different pH levels on hypopharyngeal cell lines.[18,22,23,27,28,39,51] Johnston et al detected intracellular pepsin within 1 hour of exposure.[18] Samuels et al found that the pepsin treatment of cell lines altered 397 genes linked to cancer signaling pathways, including dysregulation of MALAT1, keratin-82, and the long noncoding RNA LRP1-AS, which regulates the putative pepsin receptor LRP1.[51] Kelly et al exposed FaDu cells to nonacidic pepsin, and reported an increased rate of relative wound density, cell migration, and viability of exposed cells compared with controls, which supported an apoptotic resistance.[28] The same team reported on FaDu cell lines that pepsin altered the expression of 27 genes involved in carcinogenesis and affected the expression of 22 miRNAs that are altered in L/HSCC,[23] while the pepsin activity, pepsin-mediated cell damage, and the miRNA changes were inhibited by curcumin.[27] Other teams reported that pepsin enhanced cell proliferation and migration,[22,39] upregulated Glut-1 expression promoting glycolysis,[39] and, as under-mentioned, affected epithelial-mesenchymal transition by upregulating IL-8, Vimentin, and β-catenin while downregulating E-cadherin.[22] Finally, three studies focused on the NF-κB pathway activation through pepsin.[25,30,37] Studies reported that pepsin significantly enhanced NF-κB transcription factor activity and upregulated expression of downstream molecules similarly involved in bile acid-induced processes (eg, RELA, c-REL, Bcl-2, TNF-α, EGFR, STAT3, WNT5A, and ΔNp63), which also increased the S-phase cell fraction while decreasing the G1-phase fraction.

Animal model studies

Twelve studies used animal models to investigate the carcinogenesis potential of reflux contents. Johnston et al confirmed the human cell findings through the identification of an overexpression of Sep53 and Sep70 in porcine laryngeal tissue after exposure to pepsin, while the HSP70 expression was unchanged.[18] Chen et al did not corroborate the HSP70 preservation in a rat model exposed to several pepsin concentrations. In this study, the authors found that pepsin stimulation increases HSP70 in both laryngeal epithelial and cancer cells, inhibiting endoplasmic reticulum stress (ERS) and related apoptosis/autophagy. However, they reported that HSP70 inhibition produced differential effects: in normal cells, it suppressed glucose-regulated protein 78 (GRP78) and promoted apoptosis, while cancer cells maintained resistance to apoptosis, despite GRP78 reduction. HSP70/ERS inhibition effectively reduced tumor growth *in vivo*.[49] Another study found pepsin stimulation induced local inflammatory changes and lymphocyte infiltration in murine hypopharynx.[41]

Sasaki et al demonstrated that gastroduodenal fluids induced preneoplastic changes in mouse laryngeal mucosa, with acidic bile combinations showing the strongest effects.[42] Acidic bile significantly increased cell proliferation markers (Ki67, CK14, and ΔNp63), oncogenic p-STAT3, and altered cell adhesion molecules. It also upregulated cancer-promoting microRNAs (miR-21, miR-155, and miR-192) while suppressing tumor-suppressive miRNAs (miR-34a, miR-375, and miR-451a), with these changes correlating with NF-κB activation. The findings of this murine study supported those found in cell lines, and human tissue investigations. Seven studies exploring bile acid effects through animal model research consistently demonstrated an activation of the NF-κB pathway and related altered expression of upstream/downstream molecules.[43–48,50] Three studies additionally found bile acids promoted local micro-injury, hyperplasia, dysplasia, and neoplastic changes,[43,46,50] and three others reported bile acid-induced upregulation of oncogenic miRNAs (miR-192, miR-21, and miR-155) and downregulation of tumor

suppressor miRNAs (miR-375, miR-34a, miR-451a, miR-504, and miR-99a).[44,46,48]

DISCUSSION

The causal relationship between reflux and L/HSCC has been suggested for a long time through clinical studies investigating the prevalence and findings of GERD in patients with LSCC or HSCC.[7,9] However, meta-analyses reported controversial results, which can be attributed to the diagnostic methods used for documenting reflux, most studies considering esophageal reflux features (eg, acid exposure time, GERD complications) rather than LPRD.[7,9] Because of the lack of large-cohort studies considering pharyngeal reflux rather than esophageal reflux characteristics in L/HSCC,[8] the summarization of experimental/basic science studies makes sense to explore the causality between LPRD and L/HSCC.

The translational analysis of the findings found in the present study strongly supports that LPRD-related gastroduodenal content can promote carcinogenesis of laryngopharyngeal epithelial cells through numerous pathways, particularly the NF-κB pathway.[24,29,30,33–38] The pro-inflammatory potential of bile acids and pepsin was extensively investigated in otolaryngology, with most studies identifying micro- and macroscopic laryngeal and pharyngeal mucosal changes in patients with a demonstrated LPRD.[52–54] In benign lesions or clinical LPRD without neoplasia, pepsin has been found to reduce defense mechanisms of epithelial cells through a downregulation of mucin,[55] carbonic anhydrase,[56] Sep70,[57] HSP,[58] and genes, while promoting inflammatory infiltrate in the mucosa.[52,53,59] The present review identified common protein expression alterations in both benign and malignant conditions associated with LPRD. Importantly, the activation of the NF-κB pathway concerned both pepsin and bile acids in acidic environment,[29,33–36,38] which strengthens the importance of potential targeted therapy of NF-κB-activated molecules rather than targeting enzymes alone. Despite the identification of key mechanisms linking reflux content to L/HSCC initiation, progression, invasion, and metastatic progression, the heterogeneity across clinical and experimental studies for inclusion criteria, reflux diagnosis, investigated cell molecules, and digestive enzymes limits the establishment of a valid biomolecular model of mechanistic association between LPRD and cancer.

First, the clinical studies, including patients with LPRD with or without L/HSCC, did not perform the LPRD diagnosis through the identification of pharyngeal reflux events at the 24-hour HEMII-pH. Four studies using human tissue specimens based the LPRD diagnosis on reflux finding score and reflux symptom index,[19,20,23,25] or endoscopic findings of reflux.[24] Regarding the non-specificity of symptoms and findings, the use of reflux symptom index or endoscopic score may be associated with the inclusion of patients with laryngopharyngeal symptom-induced conditions but no LPRD (eg, allergy, chronic rhinitis, chronic rhinosinusitis, and tobacco-induced laryngitis).[60–63] This sample bias is particularly important in studies investigating mucosa inflammation signatures, which can be influenced by these other conditions. Only Johnston et al used objective LPRD diagnosis through the dual-probe pH metry, which can detect acidic LPRD.[18] This heterogeneity may be addressed in future studies considering the use of 24-hour HEMII-pH to objectify the occurrence of pharyngeal reflux events in patients with LPRD symptoms and findings.[14,15]

Second, most studies focused on pepsin and bile acids to investigate the biomolecular mechanisms underlying the development of L/HSCC. Recent studies demonstrated that elastase and trypsin may be detected in the saliva of patients with a demonstrated LPRD diagnosis at the 24-hour HEMII-pH, with only elastase demonstrating a significantly higher salivary level in LPRD patients compared with controls.[11] The potential role of elastase in the development of mucosa inflammation and related symptoms was similarly supported in patients with LPRD-induced chronic cough.[64] Because the saliva of LPRD patients is more alkaline than controls,[11,64] the investigation of trypsin and elastase, both activated in weakly acidic or alkaline pH, as promoters of carcinogenesis patterns, may bring new insights into the understanding of reflux-induced L/HSCC carcinogenesis.

Third, regarding the increased number of studies reporting the importance of tumor cell heterogeneity,[65,66] the use of organoid models of laryngeal or hypopharyngeal tumors derived from patient tumor tissue may be more consistent than cell lines or animal models, because organoid models maintain the genetic and phenotypic characteristics of the original cancer, including potential heterogeneity,[67] allowing researchers to study tumor behavior when exposed to digestive enzymes at different pH environments, testing potential therapies in a more relevant context than traditional 2D cell cultures. Organoid models are a promising alternative to traditional animal models for LPRD research. Animal models have significant limitations: quadruped-induced liquid reflux rather than the typical gaseous reflux pattern of bipedal humans, and show considerable variability in molecular expression patterns and tissue composition across laryngeal/hypopharyngeal regions both between individual animals and in comparison to humans.[68,69] The microbiome is an additional part of the peritumoral and tumoral environment, which is not considered in the current literature and particularly in both cell lines and animal model studies. The consideration of microbiome in the development of L/HSCC in LPRD patients is strengthened by a recent study demonstrating significant taxonomic-level alterations in alpha diversity (reduced Shannon index at family and genus levels in LPRD) and beta diversity (distinct community composition between LPRD and controls), with differential abundance of key taxa, including modulated *Streptococcus* species, elevated *Actinomyces* and *Abiotrophia*, and depleted *Oribacterium* and *Eubacterium nodatum* group in

LPRD patients compared with controls.[70] In this translational study, elastase, trypsin, and bile salts reported significant association with relative abundance of some bacteria, while the phylum *DTB120* (*Candidatus Ferristratum* sp) was strongly associated with the total number of pharyngeal reflux episodes. In a recent systematic review investigating the involvement of the microbiome in the development of LSCC, it has been found that *Streptococcus*, which was depleted in the LPRD group,[70] was similarly depleted in patients with LSCC,[71] and oropharyngeal and hypopharyngeal squamous cell carcinomas.[72] In summary, the current literature may suggest an important role of microbiome in the development of L/HSCC, with reflux as potential trigger of microbial alterations, and related impaired immune system modulations.[73,74] The heterogeneity across studies for these numerous points and the lack of translational large-cohort studies are the primary limitations of the present review.

CONCLUSION

Pepsin and bile acids contribute to laryngeal and hypopharyngeal carcinogenesis, invasions, and metastatic progression by disrupting defense mechanisms, cellular functions, and epigenetics. Activation of the NF-κB pathway appears to be a key mechanistic driver. Further studies considering patients with a demonstrated LPRD diagnosis, and the investigation of all digestive enzymes, are needed to elucidate the underlying mechanistic mechanisms.

Author contributions

Jerome R. Lechien, Marie Mailly, and Stephane Hans: 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.

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Declaration of Competing Interest

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