Salivary Pepsin Measurement in Laryngopharyngeal Reflux Disease: A Systematic Review of Diagnostic Accuracy and Performance

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SUMMARY: Objective. To analyze the methods used for digestive enzyme measurements in saliva of patients with laryngopharyngeal reflux disease (LPRD) and to investigate their respective diagnostic performances.

Methods. Three independent investigators conducted a PubMED, Scopus, and Cochrane Library database search for studies investigating the digestive saliva enzyme measurements in LPRD patients according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements.

Results. Of the 183 screened studies, 38 studies met the inclusion criteria (1461 females (47.5%) and 1614 males (52.5%)). The mean age of patients was 43.5 years. Two studies assessed the accuracy of salivary pepsin in pediatric populations. Twelve studies included patients with an objective LPRD diagnosis at the 24-hour hypopharyngeal-esophageal multichannel intraluminal impedance-pH testing. The lateral flow immunohistochemistry (Peptest) and ELISA were the most common approaches for measuring pepsin. Sensitivity ranged widely (27.0-93.8%) across different methods and thresholds, with Peptest showing 27.0-87.1% sensitivity and 25.0-100% specificity at ≥16 ng/mL threshold. ELISA demonstrated 20.0-93.8% sensitivity and 45.5-84.3% specificity across various cutoff values. Higher thresholds generally improved specificity at the expense of sensitivity. Multiple saliva measurements throughout the 24-hour testing period improved the sensitivity and specificity of the pepsin test. Only three studies considered the measurement of other digestive enzymes, primarily bile salts, as biomarkers of LPRD.

Conclusion. The methods of salivary pepsin collection and measurement substantially influence its diagnostic performance. Future comparative studies are needed to determine the most accurate methodological approach and to establish consensus guidelines for salivary pepsin and other digestive enzyme measurements in LPRD diagnosis through standardized collection, storage, and measurement protocols.

Key Words: Otolaryngology–Otorhinolaryngology–Voice–Laryngopharyngeal reflux–Pepsin.

INTRODUCTION

The consensus of the International Federation of Otorhinolaryngological Societies (IFOS) defined laryngopharyngeal reflux disease (LPRD) as a disease of the upper aerodigestive tract resulting from the direct and/or indirect effects of gastroduodenal contents of reflux, inducing morphological and/or neurological changes in the upper aerodigestive tract.[1] The current gold standard for diagnosing LPRD is the 24-hour Hypopharyngeal-Esophageal Multichannel Intraluminal Impedance-pH monitoring (HEMII-pH), which documents acid, weakly acid, and alkaline pharyngeal reflux events in patients with LPRD symptoms (eg, globus pharyngeus sensation, throat clearing, sticky mucus, halitosis, chest pain, cough, nausea, and regurgitations) and findings (eg, oropharyngeal wall erythema, laryngopharyngeal sticky mucus, tongue tonsil hypertrophy, posterior commissure erythema, and hypertrophy).[1] The primary limitations of HEMII-pH remain its invasiveness, cost, and limited availability in some clinical settings.[2] Some noninvasive alternative diagnostic approaches have therefore been developed, including salivary pepsin detection. Peptest was developed in 2007 by RD Biomed (Castle Hill, UK) and represents the most widely used device to measure salivary pepsin. Peptest is based on lateral flow immunohistochemistry with two monoclonal human pepsin antibodies—one for detection and one for capturing pepsin.[3] An increasing number of studies have evaluated the performance of salivary pepsin detection in LPRD, reporting varying levels of sensitivity and specificity. These discrepancies may be attributed to the heterogeneity of methodologies, including Western blot, Enzyme-Linked Immunosorbent Assay (ELISA), and Peptest itself. To date, only the Peptest exists as nonexperimental, clinical, and friendly-use device for measuring pepsin in clinical practice. Other approaches are experimental and require lab analyses.

This systematic review aims to investigate the methods used for digestive enzyme measurements in saliva of patients with laryngopharyngeal reflux disease (LPRD) and to investigate their respective diagnostic performances.

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MATERIALS AND METHODS

This review was conducted by three independent investigators (J.R.L., A.T., and A.H.) with the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) checklist.[4] The criteria for considering studies were based on the population, intervention, comparison, outcome, timing, and setting (PICOTS) framework.[5]

Type of studies

Investigators considered controlled/uncontrolled prospective, retrospective, cross-sectional, diagnostic accuracy, and pilot studies published between January 2000 and March 2025 in English-language peer-reviewed journals. Studies had to investigate accuracy and methods of pepsin measurements in the saliva of LPRD patients. Other enzymes were not considered regarding the very low number of studies assessing the other digestive enzymes. Case reports, letters, and comments were excluded.

Population

The criteria used for the LPRD diagnosis were extracted. The LPRD diagnosis was based on the findings of the Dubai consensus.[1] Thus, the diagnosis of LPRD was confirmed if patients had more than one hypopharyngeal reflux event at the 24-hour HEMII-pH. The diagnosis was suggested but not confirmed for patients who underwent oropharyngeal pH monitoring, dual- or triple-probe pH monitoring with or without pharyngeal pH sensor. Specifically, patients with more than one pharyngeal reflux events at the dual- or triple-probe pH monitoring (without impedance sensor) were considered as patients with acid LPRD.[1] The LPRD diagnosis was suspected but not confirmed for patients included through the use of patient-reported outcomes questionnaires (eg, reflux symptom index (RSI),[6] reflux symptom score (RSS)[7]) and validated sign instruments (eg, reflux finding score (RFS),[8] reflux sign assessment (RSA)[9]). Patients with LPRD symptoms and positive gastroesophageal reflux disease (GERD) diagnosis according to the Montreal and Lyon consensus[10] were suspected of having LPRD diagnosis. In this review, both patients with confirmed or suspected LPRD were included.

Intervention and comparison

Intervention consisted of the measurement of salivary pepsin in suspected or confirmed LPRD patients. No criteria related to therapeutic intervention were considered.

Outcomes

The primary outcomes included the diagnosis method of LPRD (eg, 24-hour HEMII-pH, pH monitoring, or clinical and empirical approaches), and the details related to the pepsin measurement approaches (eg, Western blot, ELISA, fibrinogen digestion assay, and Peptest). Because these methods differ in their analytical approaches and ability to detect and quantify pepsin, a critical analysis of the method features was performed by two investigators, both specialized in biomedical science, to provide clinical insights. The specificities of methods were considered. Specifically, Western blot was used to detect and quantify pepsin at the molecular level,[11] while ELISA provided accurate quantification of pepsin concentration in saliva.[12] The fibrinogen digestion assay assesses pepsin's proteolytic activity by measuring fibrinogen degradation. The Peptest, a lateral flow immunoassay, enables rapid, real-time detection of pepsin in saliva samples.

The secondary outcomes included study design, number of patients, gender ratio, age, and diagnosis criteria. Data on comorbidities were also extracted when available, as well as information on whether ongoing treatments, such as proton pump inhibitors (PPIs) or other anti-reflux therapies, were considered in the inclusion and exclusion criteria of the studies.

Time and setting

There were no strict criteria for time and setting in these studies.

Search strategy

Three investigators independently conducted PubMed, Cochrane Library, and Scopus science database searches for relevant peer-reviewed publications related to methodologies for measuring pepsin in the saliva of LPRD patients. The following keywords were used: "Reflux," "Laryngopharyngeal," "Gastroesophageal," "Pepsin," "Enzymes," and "Saliva." The studies reporting database abstracts, available full texts, or titles containing the search terms were considered. The research findings were reviewed for relevance, and the reference lists of these articles were examined for additional pertinent studies. The included studies were analyzed for the number of patients, inclusion and exclusion criteria, demographics, and outcomes.

RESULTS

Of the 183 screened studies across three electronic databases, 38 studies met the inclusion criteria (Figure 1). There were 30 prospective studies,[13–41] including cohort studies (n = 7),[15,16,19,22,24,28] observational studies (n = 8),[13,18,26,36,42–45] cross-sectional studies (n = 6),[3,16,32,39,41,46] diagnostic accuracy study (n = 1),[47] controlled prospective studies (n = 6),[14,25,30,33,35,38] uncontrolled studies (n = 2),[20,48] and preliminary pilot studies (n = 2).[34,49] The review included data from 1461 females (47.5%) and 1614 males (52.5%), respectively (Table 1). The mean age of patients was 43.5 years (n = 3075). Of the 38 included studies, two studies considered pediatric populations.[39,41]

Clinical and diagnostic outcomes

The clinical outcomes and diagnosis approaches are reported in Table 1. Thirteen diagnosis methods were identified in studies, the most common being symptoms and/or sign

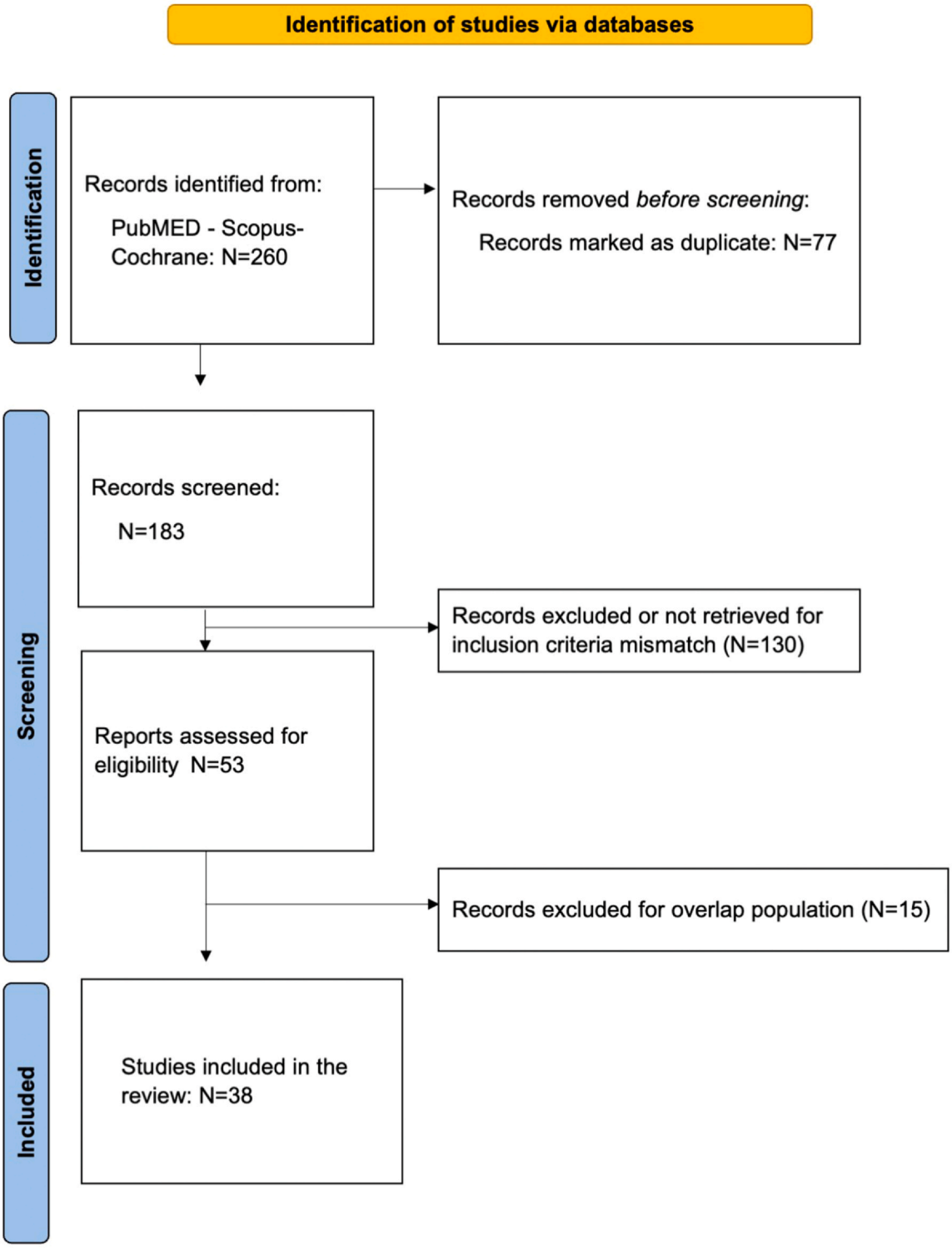

FIGURE 1. PRISMA flowchart.

evaluation. Among them, validated patient-reported outcome questionnaires (RSI and RFS) were used in 17 (44.7%) studies with adherence to the initial thresholds defined by Belafsky et al for suggesting the LPRD diagnosis (RSI > 13 and RFS > 7) in 14 studies.[3,13,15,17,20,23,29,31,34,40,44,45,47,49] Objective examinations were used in 24 studies, including 24-hour HEMII-pH (n = 12),[14,16–19,21,22,24,25,30,42,48] multichannel intraluminal impedance-pH (MII-pH) monitoring (n = 8),[26–28,31,33,35,39,41] single-[36,37] dual-[32] probe pH metry (n = 3), and wireless pH metry (n = 1).[38] There was a myriad of diagnostic criteria for considering LPRD at the pH/impedance testing (Table 1).

TABLE 1.
Demographic and Clinical Findings

Reference	Study design	Sample size	Age	Female/ Male	Reflux diagnosis method
Guo (2024)[13]	Prospective observational	67 sLPR	39.5	15/52	RSS >13
Lechien (2024)[14]	Prospective controlled	67 LPR 57 CT	NP	LPR 34/43	>1 Pharyngeal reflux at 24-h HEMII-pH monitoring
Yun (2023)[15]	Prospective cohort	25 sLPR	47.1	9/16	RSI ≥14 and RFS
Zhang (2023)[17]	Prospective Cross-sectional	77 sGER 12 CT	LPR 42.2 CT 48.0	39/38 9/3	GERDQ, RSI, HRM and 24-h dual MII-pH monitoring
Zhang (2023)[48]	Prospective uncontrolled	125 sLPR 28 CT	LPR 56.6 CT 39.8	24/101 14/14	24-h HEMII-pH monitoring
Kang (2022)[18]	Prospective observational	48 sLPR	54.9	35/13	>1 Reflux episode at 24-h MII-pH monitoring
Wang (2022)[22]	Prospective cohort	138 sLPR	NP	26/112	≥1 Pharyngeal event at 24-h HEMII-pH monitoring
Yu (2022)[42]	Observational	40 sLPR	LPR 47.2 CT 40.9	12/18 4/6	24-h MII-pH monitoring and RSI >13 and/or RFS >7
Casciaro (2022)[43]	Observational	30 sLPR	40.6	24/6	RSI >13
Zhang (2021)[20]	Prospective uncontrolled	204 sLPR	38.8	39/165	RSI >13 or RFS >7
Im (2021)[44]	Observational	114 CT	NP	35/79	RSI ≥13
Guo (2021)[45]	Observational	120 sLPR	LPR 44.3 CT 44.7	16/34 22/58	RSI >13 and RFS >7
Zelenik (2021)[21]	Prospective	46 sLPR	49*	30/16	>1 Pharyngeal event at 24-h HEMII-pH monitoring
De Corso (2021)[26]	Prospective observational	62 sLPR	55.7	24/38	>1 Reflux episode, percentage of acid exposure time (pH <4) >0.5% at 24-h MII-pH monitoring
Bozzani (2020)[49]	Noninterventional pilot	86 sLPR 59 CT	LPR 53.7 CT 40.5	49/37 29/30	RSI ≥13
Divakaran (2020)[23]	Prospective	120 sLPR	41	67/53	RFS >7 and RSI >13
Zhang (2020)[19]	Prospective cohort	35 sLPR	55.5*	14/16	≥2 Pharyngeal events, ≥6 proximal events at 24-h HEMII-pH monitoring
Bobin (2020)[16]	Prospective cohort	65 LPR	56.0	45/20	>1 Proximal events at 24-h HEMII-pH monitoring
Klimara (2020)[25]	Prospective case-control	39 sLPR	LPR 34 CT 49	17/9 9/4	>1 Pharyngeal event or >40 proximal events at 24-h HEMII-pH monitoring
Weitzendorfer (2019)[24]	Prospective cohort	70 sLPR	54.4	40/30	>73 Reflux at 24-h HEMII-pH monitoring
Bor (2019)[27]	Prospective	20 sLPR	40.3	8/12	pH <4 for 5% of the time at 24-h MII-pH monitoring
Barona-Lleo (2019)[47]	Diagnosis accuracy	221 CT	48.5	136/85	RSI ≥13
Jung (2019)[28]	Prospective cohort	50 sLPR	51.7	32/18	≥1 Reflux events at 24-h MII-pH monitoring
Barona-Lleo (2018)[29]	Prospective	142 sLPR	NP	89/53	RSI >13

TABLE 1 (*Continued*)

Reference	Study design	Sample size	Age	Female/ Male	Reflux diagnosis method
Yadlapati (2017)[40]	Prospective blinded cohort	59 sLPR	35.8	LPR 10/8 LPR + GER 13/5 CT 14/3	RSI >13 and GERDQ >8
Na (2016)[30]	Prospective case-control	50 LPR 12 CT	LPR 51.7 CT 37.8	32/18 8/4	≥1 Reflux events at 24-h HEMII-pH monitoring
Dy (2016)[41]	Prospective cross-sectional	50 sLPR	8.7	16/34	24-h MII-pH monitoring and PedsQL/PGSQ
Spyridoulias (2015)[31]	Prospective	78 sLPR	54.6	59/19	RFS >7, RSI >13, and 24-h MII-pH monitoring
Ocak (2015)[32]	Prospective cross-sectional	20 sLPR	NP	12/8	24-h dual-probe esophageal pH monitoring
Sereg-Bahar (2015)[33]	Prospective comparative	28 sLPR 48 CT	47 52.4	19/9 26/22	HRM and combined MII and 24-pH monitoring
Hayat (2015)[35]	Prospective blinded controlled	134 sLPR 104 CT	49.7 30.7	55/45 62/49	RDQ and 24-h MII-pH monitoring
Fortunato (2015)[39]	Prospective cross-sectional	90 LPR 43 CT	3* 4*	36/54 21/22	24-h MII-pH monitoring and GERDQ
Hayat (2014)[46]	Cross-sectional	21 sLPR 10 CT	51 26	sLPR 15/6	RSI >13 and RFS >7
Yuksel (2012)[38]	Prospective blinded controlled	58 sGER 51 CT	50* 46*	20/38 20/31	EGD and 48-h wireless pH monitoring
Wang (2010)[3]	Cross-sectional	56 sLPR 15 CT	37.7 25.0	29/27 6/9	RSI >13 and RFS >7
Kim (2008)[36]	Prospective observational	40 sGER	45	26/14	pH <4 for 4% of the time at 24-h esophageal pH monitoring
Printza (2007)[34]	Prospective pilot	9 sLPR 2 CT	38* 35.5*	6/3 2/0	RSI and RFS
Potluri (2003)[37]	Prospective	16 sGER	49.5	13/03	24-h esophageal pH monitoring

*Median age.

Abbreviations: CT, control; dual-probe pH monitoring, dual pH sensor pH monitoring; EGD, esophagogastroduodenoscopy; GERDQ, Gastroesophageal Reflux Disease Questionnaire; HEMII, hypopharyngeal-esophageal multichannel intraluminal impedance; HRM, high-resolution manometry; MII-pH, multichannel intraluminal impedance-pH monitoring (without pharyngeal sensor); N.S., not specified; PedsQL, Pediatric Quality of Life Questionnaire; PGSQ, Pediatric Gastroesophageal Reflux Disease Symptom and Quality of Life Questionnaire; RDQ, Reflux Disease Questionnaire; RFS, Reflux Finding Score; RSI, Reflux Symptom Index; RSS, Reflux Symptom Score; sGER, suspected gastroesophageal reflux; sLPR, suspected laryngopharyngeal reflux.

Enzyme measurement outcomes

Sampling protocols

Pepsin was the most measured enzyme (Table 2). The other digestive enzymes that were searched in the saliva additionally to pepsin included bile salts,[14,26,33] elastase,[14] and lipase.[14] Pepsin was measured in saliva through variable sampling protocols. In most studies, samples were collected in the morning, fasting, or 1-2 hours postprandially through a single collection. Guo et al[45] collected three samples per patient (fasting morning, post lunch, and post dinner). Other studies collected samples hourly throughout the day (eg, from morning to 6 PM).[13,21,49,51] Some studies instructed participants to collect saliva immediately following symptom onset, often using citric acid-preloaded tubes to preserve enzyme activity.[16,17,19,27,32,34,36,49] Overall, the timing of saliva collection—fasting, postprandial, or symptom-triggered—varied widely across studies, contributing to methodological heterogeneity.

Analytical methods

The lateral flow immunochromatographic assay (Peptest) was the most used method for measuring salivary pepsin levels (n = 23; 60.5%; Table 2). Peptest uses two monoclonal antibodies specific to pepsin and provides a semi-quantitative result via a lateral flow cassette read by a Cube Reader (1-500 ng/mL range, ± 15 minutes). ELISA, which provides more precise quantitative analysis while requiring laboratory equipment and more time, was the second most used approach (n = 11; 28.9%). Western blotting and fibrinogen digestion assays were used in three studies.[34,36,37] As reported in Table 2, saliva was collected in acidified tubes containing citric acid 0.01-0.1 M to preserve pepsin integrity, maintaining pH around 2-4 to preserve pepsin activity. Samples were then stored at 4 °C, −20 °C, or −80 °C, and remained stable for several days to 6 months without significant degradation.[18]

TABLE 2.
Methodological and Biomedical Outcomes

Reference	Sample time	Collection device	Storage °C	Analysis time	Analysis method	Analysis details	Cutoff (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)
Guo (2024)[13]	Each hour (8 AM to 6 PM)	NP	NP	NP	Peptest	NP	NP	NP	NP	NP	NP
Lechien (2024)[14]	Fasting	0.5 mL of 0.01 M citric acid pH 2.5	−20	NP	Bile acids: Assay kit Elastase: ELISA Lipase: ELISA Cholesterol: CHOL2 assay	NP	NP	NP	NP	NP	NP
Yun (2023)[15]	Fasting	0.5 mL of 0.01 M citric acid	NP	NP	Peptest	Centrif. 4k rpm—5 min; 80 µL sup. + 240 µL migration buffer	≥16	NP	NP	NP	NP
Zhang (2023)[17]	Fasting Post lunch Post dinner During symptoms	0.5 mL of 0.01 M citric acid pH 2.5	4	Within 7d	Peptest	Centrif. 4k rpm—5 min; 80 µL sup. + 240 µL migration buffer	122.65	100	66.7	NP	NP
Zhang (2023)[48]	Each hour (morning to 6 PM)	NP	NP	NP	Peptest	NP	>45 MTPSPT >45 Fasting	90.5 45.0	75.0 75.0	95.9 90.6	61.5 20.8
Kang (2022)[18]	Fasting	1 mL of 0.1 M citric acid pH 2.5	4	T0/1w/2w/ 1m/ 3m/6m	ELISA	Centrif. 1 rpm—20 min—4 °C; sup.; ELISA (pepsin)	>0	83.8	45.5	NP	NP
Wang (2022)[22]	Each hour (7 AM to 6 PM)	0.5 mL of 0.01 M citric acid	NP	NP	Peptest	NP	>45 MTSPT >45 Fasting test	86.6 38.4	80.8 84.6	95.1 91.5	58.3 24.2
Yu (2022)[42]	Fasting (after oral rinsing)	0.5 mL of 0.01 M citric acid	−80	NP	ELISA	Centri. 3k rpm—15 min—4 °C; sup.; ELISA (pepsin)	219.5	93.3	70.0	90.3	77.8
Casciaro (2022)[43]	1 h after eat/ drink	1 mL of 0.01 M citric acid	NP	NP	Peptest	Centri. 400 rpm —5 min—RT; 80 µL sup. + 240 µL migration buffer	NP	NP	NP	NP	NP
Zhang (2021)[20]	Each hour (7 AM to 6 PM)	NP	NP	NP	Peptest	NP	≥45 MTPSPT >45 Fasting	76.4 37.9	85.9 92.2	92.2 91.9	62.5 40.4
Im (2021)[44]	Fasting	10 mL of distilled water	4	Within 2 d	Pepsin/ pepsinogen kit	Samples + 1/10 pepsin substrate —10 min —37 °C; read 328 nm/418 nm	> 0.64 x 10^3 pmol/mg	51.4	74.7	NP	NP

TABLE 2 (*Continued*)

Reference	Sample time	Collection device	Storage °C	Analysis time	Analysis method	Analysis details	Cutoff (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)
Guo (2021)[45]	Fasting 1 h post lunch 1 h post dinner	0.5 mL of 0.01 M citric acid pH 2.5	4	NP	NP	NP	≥33	82.5	74.0	NP	NP
Zelenik (2021)[21]	Fasting	0.5 mL of 0.01 M citric acid	4	T0	Peptest	Centrif. 4k rpm—5 min; 80 µL sup. + 240 µL migration buffer	≥16	27.0	63.0	40.0	48.0
De Corso (2021)[26]	Fasting 1 h post lunch 1 h post dinner	0.5 mL of 0.01 M citric acid pH 2.5	−80	NP	Bile acids: Colorimetric assay Pepsinogen I-II: ELISA	NP	Bile acids: 0.4 µmol/L Pepsinogen I: 0.1 Pepsinogen II: 0.1	87.0 20.8 50.0	58.3 75.0 66.7	80.0 62.5 75.0	70.0 32.1 40.0
Bozzani (2020)[49]	Continued symptoms Episodic symptoms	0.5 mL of 0.01 M citric acid	4	Within 5 d	Peptest	NP	25	NP	NP	NP	NP
Divakaran (2020)[23]	1 h post meals	0.5 mL of 0.01 M citric acid pH 2.5	4	Within 1 w	Peptest	Centrif. 4k rpm—5 min; 80 µL sup. + 240 µL migration buffer	NP	NP	NP	NP	NP
Zhang (2020)[19]	Fasting Post lunch Post dinner During symptoms	0.5 mL of 0.01 M citric acid	4	NP	Peptest	NP	≥16 ≥75	76.9 57.7	25.0 75.0	87.0 93.8	14.3 21.4
Bobin (2020)[16]	30 min before/after symptoms	0.5 mL of 0.01 M citric acid	−20	NP	Peptest	Centrif. 4k rpm—5 min; 80 µL sup. + 240 µL migration buffer	≥16	NP	NP	NP	NP
Klimara (2020)[25]	Fasting 1 h post meals	NP	4	NP	ELISA	NP	> 1	NP	NP	NP	NP
Weitzendorfer (2019)[24]	Fasting 1 h post lunch 1 h post dinner	0.5 mL of 0.01 M citric acid	4	Within 2 d	Peptest	Centrif. 4k rpm—5 min; 80 µL sup. + 240 µL migration buffer	≥16 50 100 150 216	85.4 78.1 68.3 53.7 41.5	27.6 41.4 58.6 69.0 86.2	62.5 65.3 70.0 71.0 81.0	57.1 57.1 56.7 51.3 51.0
Bor (2019)[27]	During symptoms	0.5 mL of 0.01 M citric acid pH 2.2-2.8	4	NP	Peptest	Centrif. 4k rpm—5 min; 80 µL from sup. + 240 µL migration buffer	> 16	87.1	67.5	NP	NP

TABLE 2 (*Continued*)

Reference	Sample time	Collection device	Storage °C	Analysis time	Analysis method	Analysis details	Cutoff (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)
Barona-Lleo (2019)[47]	Fasting 1 h post meal	0.5 mL of 0.01 M citric acid	4	Within 7 d	Peptest	Centrif. 4k rpm—5 min; 80 μL from sup. + 240 μL migration buffer	≥16 Fasting ≥16 Double Peptest	40.0 48.0	97.5 95.0	96.6 94.8	27.0 48.7
Jung (2019)[28]	Fasting	0.5 mL of 0.01 M citric acid pH 2.5	−80	Within 2 m	ELISA	Centrif.1k g—15 min—4 °C; sup.; ELISA (pepsin)	NP	NP	NP	NP	NP
Barona-Lleo (2018)[29]	Fasting 1 h post meal	0.5 mL of 0.01 M citric acid	4	Within 7 d	Peptest	Centrif. 4k rpm—5 min; 80 μL from sup. + 240 μL migration buffer	16	NP	NP	NP	NP
Yadlapati (2017)[40]	NP	0.5 mL of 0.01 M citric acid pH 2.5	4	Within 1 w	Peptest	Centrif. 4k rpm—5 min; sup. + migration buffer	> 16	NP	NP	NP	NP
Na (2016)[30]	Fasting 1 h post meals During symptoms	0.5 mL of 0.01 M citric acid pH 2.5	−80	Within 2 m	ELISA	Centrif. 1k g—15 min—4 °C; sup.; ELISA (pepsin)	NP	NP	NP	NP	NP
Dy (2016)[41]	Fasting	0.5 mL of 0.01 M citric acid	4	Within 1 w	Peptest	Centrif. 4k rpm—5 min; 80 μL from sup. + 240 μL migration buffer	> 74	17.0	100	100	57.0
Spyridoulias (2015)[31]	Post dinner	0.5 mL of 0.01 M citric acid	NP	Within 36 h	Peptest	Centrif.; sup. + migration buffer	25	78.0	53.0	NP	NP
Ocak (2015)[32]	During symptoms	Citric acid	NP	NP	Peptest	Centrif. 4k rpm—5 min; 80 μL from sup. + 240 μL migration buffer	16	33.0	100	100	14.2
Sereg-Bahar (2015)[33]	2 h post dinner	NP	−20	NP	Bile acids: Enzyme kit Pepsin: ELISA	NP	NP	NP	NP	NP	NP
Hayat (2015)[35]	Fasting 1 h post lunch 1 h post dinner	0.5 mL of 0.01 M citric acid	4	Within 2 d	Peptest	Centrif. 4k rpm—5 min; 80 μL from sup. + 240 μL migration buffer	> 16 > 210	77.6 44.2	63.2 96.3	58.4 95.2	80.4 36.5
Fortunato (2015)[39]	Before meals 30 min post meals	0.5 mL of 0.01 M citric acid pH 2.5	−20	NP	ELISA	NP	10 50	60.0 20.0	55.7 84.3	27.9 26.7	83.0 78.7

TABLE 2 (*Continued*)

Reference	Sample time	Collection device	Storage °C	Analysis time	Analysis method	Analysis details	Cutoff (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)
Hayat (2014)[46]	Fasting At midday 17 h 1 hour post dinner Bedtime	0.5 mL of 0.01 M citric acid	4	Within 2 d	Peptest	Centrif. 4k rpm—5 min; 80 µL from sup. + 240 µL migration buffer	25	NP	NP	NP	NP
Yuksel (2012)[38]	During endoscopy	0.5 mL of 0.01 M citric acid pH 2.5	4	Within 1 w	Peptest	Centrif. 4k rpm—5 min; sup. + migration buffer	50	50.0	92.0	85.0	68.0
Wang (2010)[3]	NP	NP	NP	NP	ELISA	Diluted 0.1% DDT—10 min—37 °C; Centrif. 5k rpm—15 min—4 °C; ELISA (pepsin)	0.1081	93.8	80.0	NP	NP
Kim (2008)[36]	Fasting Symptom Bedtime	0.5 mL of 0.1 M citric acid pH 2.5	4	NP	Western blot	Centrif. 20kg—30 min—4 °C; sup. + anti-pepsin	NP	89.0	68.0	44.0	95.0
Printza (2007)[34]	Every 30 min (24 h) During symptoms	NP	4	NP	Fibrinogen digestion	Plates incubation humid O/N; read 12 h; clear zone = pepsin activity	/	NP	NP	NP	NP
Potluri (2003)[37]	Every 2 h	NP	NP	NP	Fibrinogen digestion	1.25% agarose + bovine fibrinogen (1 mg/mL); acid 0.12 N HCl; Clearing = pepsin activity	/	63.0	92.3	75.0	100

Notes: All samples collected in standard tubes unless otherwise specified.
Abbreviations: Centrif., centrifugation; MT, multiple test; MTPSPT, multi-time point salivary pepsin test; NPV, negative predictive value; N.S., not specified; O/N, overnight; PPV, positive predictive value; SEN, sensibility; SPE, specificity; Sup., supernatant; T0, immediately.

TABLE 3.
Performance Summary Findings

Measurements and methods	Ranges				References
	SEN	SPE	PPV	NPV	
Peptest					
≥16 ng/mL	27.0-87.1	25.0-100	40.0-100	14.2-80.4	19,21,24,27,31,32,35,47
≥45 ng/mL	37.9-90.5	41.4-92.2	65.3-95.9	20.8-68.0	20,22,24,38,48
≥75 ng/mL	17.0-57.7	75.0-100	93.8-100	21.4-57.0	19,24,41
≥100 ng/mL	68.3-100	58.6-66.7	70.0	56.7	17
≥150 ng/mL	44.2-53.7	69.0-96.3	71.0-95.2	36.5-51.3	24,35
ELISA					
≥0.1 ng/mL	93.8	80.0	NP	NP	3
≥1 ng/mL	83.8	45.5	NP	NP	18
≥10 ng/mL	60.0	55.7	27.9	83.0	39
≥50 ng/mL	20.0	84.3	26.7	78.7	39
≥219.5 ng/mL	93.3	70.0	90.3	77.8	42
Western blot	89.0	68.0	44.0	95.0	36

Abbreviations: NPV, negative predictive value; PPV, positive predictive value; SEN, sensibility; SPE, specificity.

Thresholds and diagnostic performance

There was substantial heterogeneity across studies for the cutoff values to consider a positive salivary sample for pepsin measurement. The conventional threshold for Peptest was 16 ng/mL, based on the assay's lower detection limit. However, other thresholds were considered in many studies, including 1 ng/mL,[25] 25 ng/mL,[31,49] 33 ng/mL,[45] 45 ng/mL,[20,22,48] 50 ng/mL,[38,39] 74 ng/mL,[41] 75 ng/mL,[19] 100 ng/mL, 122.65 ng/mL,[17] 210 ng/mL,[35] and 219.5 ng/mL[42] (Tables 2 and 3). Based on these thresholds, the diagnostic accuracy of salivary pepsin measurements was reported in 23 studies (Table 2).[3,17–22,24,26,27,31,32,35–39,41,42,44,45,47,48] Depending on time of samples, methods, and thresholds, sensitivity and specificity substantially varied across studies (Table 3). Importantly, the minimum value of the range of specificity of Peptest progressively increased when considering an increase of the threshold, reaching 69.0% for cutoff of ≥150 ng/mL. The progressive reduction of the superior values of range of negative predictive value substantially decreased from 80.4% to 51.3% with the increase of cutoffs. These trends were reported in the study of Weitzendorfer et al[24] who evaluated multiple thresholds (≥16, 50, 100, 150, and 216 ng/mL), confirming that increasing the threshold improved specificity but reduced sensitivity. The number of studies using ELISA and Western blot was too low to provide ranges of sensitivity, specificity, and predictive values (Table 3). Note that Im et al used an alternative approach to Peptest, Western blot, and ELISA (pepsin/pepsinogen concentration kit), reporting broadly lower sensitivity (63.0%) and higher specificity (92.3%) compared with other methods.[44] Similarly, Potluri et al based their analysis on fibrinogen digestion methods, leading to specific sensitivity (63.0%), specificity (92.3%), positive (75.0%), and negative (100%) predictive values.[37] The use of multiple samples per day improved detection rates. Zhang et al[20] showed a sensitivity reaching 76.4% with multiple samples, while Wang et al[50] demonstrated a significant increase of sensitivity to 86.6% using 45 ng/mL cutoff, outperforming single fasting samples. Overall, specificity was often found to be higher than sensitivity, particularly when higher concentration thresholds were used or when results were compared with objective reference methods.

Other salivary enzymes and biomarkers

A few studies investigated alternative salivary biomarkers. Sereg-Bahar et al[33] measured both salivary bile salts and pepsin, reporting that bile acids were about three times higher in LPRD patients compared with controls. Lechien et al[14] recently analyzed salivary digestive biomarkers in LPR patients, including pH, pancreatic elastase, bile salts, cholesterol, and gastric/pancreatic lipases. They found significantly elevated levels of salivary elastases (51.65 vs 25.18 μg/mL) in LPRD patients. In this study, the salivary cholesterol was identified as an important biomarker of LPRD, with significant higher concentration of salivary cholesterol in controls versus LPRD patients. However, the authors did not identify significant differences in the levels of salivary pepsin ELISA of LPRD and controls.

DISCUSSION

The search for noninvasive diagnostic approaches for LPRD is important regarding the limited availability, cost, and tolerability issues of 24-hour HEMII-pH.

This systematic review summarizes the literature findings dedicated to the use of salivary digestive enzyme measurements for LPRD diagnosis through a critical biomedical and methodological analysis of the collection, detection, and measurement methods. The substantial heterogeneity across studies limits the establishment of clear performance values of all methods, including lateral flow immunohistochemistry (Peptest), Western blot, and ELISA. Several factors influencing the accuracy of salivary pepsin measurements were identified in this systematic review.

First, the methods for measuring salivary pepsin can significantly influence the results. Western blot, which is a qualitative immunoblot technique, can detect pepsin protein with high sensitivity. For example, Kim et al[36] reported 89% sensitivity of salivary measurements in patients with GERD diagnosis, and no confirmed LPRD. However, the method is time-consuming, nonquantitative, and requires specialized laboratory expertise, which limits its usefulness for routine clinical diagnosis.[51] ELISA, which is a quantitative antibody-based assay, is overall associated with high analytical sensitivity (detecting levels as low as 1-25 ng/mL),[52] which corresponds to lower detected pepsin levels than Peptest. The superiority of ELISA over other methods may be indirectly suggested in the study of Wang et al,[3] who found 93.8% sensitivity at a 0.1081 ng/mL cutoff, which highlights the lower detection threshold.[54] From a theoretical standpoint, ELISA can outperform the Peptest, but, similarly to Western blot, is laboratory equipment dependent, costly, and time-consuming.[53] The cost and time-consuming limitations of both ELISA and Western blot led to the development of Peptest, which is a rapid immunoassay based on monoclonal antibodies. It is user-friendly, but semiquantitative. Its detection limit (16 ng/mL) is higher than ELISA, meaning that Peptest may miss lower pepsin levels,[53] which can explain its moderate sensitivity.[54] Recent literature underscores the importance of standardizing ELISA protocols and cutoff values, suggesting that ELISA-based quantification is currently the most robust approach, despite its practical limitations.[52]

Second, some studies collecting multiple saliva samples throughout a 24-hour testing period suggested potential physiological variability of salivary pepsin concentrations. Guo et al investigated the variability of salivary pepsin levels in 67 patients with suspected LPRD through a lateral flow device.[13] They found that samples collected before morning oral hygiene and breakfast and in the evening demonstrated a higher rate of pepsin positivity compared with other time points. In the same vein, Wang et al collected multiple saliva samples in 138 LPRD patients.[22] The authors reported positive pepsin detection rates in 47 patients in the morning (sensitivity of 38.4% and specificity of 84.6%), while 102 patients were considered as positive when considering multiple saliva collections per day (sensitivity of 86.6% and specificity of 80.8%). These two studies supported the influence of the timing of saliva collection on the accuracy of the Peptest. The salivary pepsin levels could be influenced by the global severity of LPRD at the 24-hour HEMII-pH rather than both number and duration of pharyngeal reflux events, the diet, and lifestyle. According to 24-hour HEMII-pH studies, pharyngeal reflux events primarily occur daytime, upright, after meals, and may vary significantly between individuals,[55–58] depending on diet and lifestyle habits. In 2021, based on an objective refluxogenic diet scoring system, Lechien et al demonstrated that the saliva pepsin concentration during the 24-hour HEMII-pH testing period was significantly associated with foods and beverages consumed during the testing period and the evening dinner ($r = 0.973$).[58] Despite positive association with diet scores, the level of salivary pepsin should not be correlated with the number and duration of pharyngeal reflux events preceding the saliva collection. The lack of association between pharyngeal reflux events and the post-event salivary pepsin concentration may be attributed to the internalization of pepsin into the laryngopharyngeal cells,[59] limiting the accuracy of the saliva/sputum concentration measurements.

Third, it has been recently suggested that pepsin and other refluxate digestive enzymes may be degraded by other enzymes, which could interfere with their stability and detection.[60] The biochemical instability of digestive enzymes in the upper aerodigestive tract mucosa could explain the variability observed across analysis methods and sampling times. In the present review, other digestive enzymes were investigated in only three studies.[14,26,33] Lechien et al[14] conducted a controlled study comparing the salivary enzyme findings of 67 patients with LPRD and 57 asymptomatic individuals. Salivary elastase and cholesterol concentrations were found to differ significantly between LPRD patients and asymptomatic subjects,[14] with the predictive value of elastase in LPRD symptoms (chronic cough) being supported in another recent study.[60] Cholesterol,[14] bile acids,[33] and elastase[14,60] were identified as potential biomarkers of LPRD and should be considered for future development of noninvasive salivary detection systems. The consideration of such enzyme deposit makes sense regarding the nonspecificity of symptoms and findings associated with LPRD and other common otolaryngological conditions, such as allergy, chronic rhinosinusitis, laryngopharyngeal hypersensitivity, glottic insufficiency, and drug-induced laryngitis. Moreover, GERD commonly reports correlation between acid liquid reflux and esophageal symptoms, while for LPRD, it remains difficult to have demonstrated correlation between gaseous reflux events and symptoms.[57] This point strengthens the need to develop salivary device measuring the levels of all enzymes that may potentially lead to oral and laryngopharyngeal mucosa injuries, and related symptoms and findings. Concerning the detection of pharyngeal reflux events at the HEMII-pH, further studies are needed to establish potential thresholds considering the catheter characteristics and sensitivity.[61]

The heterogeneity across studies regarding LPRD diagnostic criteria, pepsin collection, storage, and measurement methods is the primary limitation of this review, constraining the ability to draw valid conclusions about performance findings. The lack of consideration of other digestive enzymes in the development of noninvasive diagnostic approaches is an additional limitation. Despite these limitations, the biomedical and methodological analysis provided in this review may offer valuable insights for conducting future studies assessing the accuracy of salivary digestive enzymes in LPRD diagnosis.

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CONCLUSION

The methods of salivary pepsin collection and measurement substantially influence its diagnostic performance. Future comparative studies are needed to determine the most accurate methodological approach and to establish consensus guidelines for salivary pepsin and other digestive enzyme measurements in LPRD diagnosis through standardized collection, storage, and measurement protocols.

Author contributions

Apolline Hiernaux: 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. **Anne Trelcat**: 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**: 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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