Esophagus
https://doi.org/10.1007/s10388-020-00714-z

ORIGINAL ARTICLE

Clinical utility of salivary pepsin measurement in patients with proton pump inhibitor-refractory gastroesophageal reflux disease symptoms: a prospective comparative study

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Received: 2 August 2019 / Accepted: 12 January 2020

Abstract

Background Salivary pepsin measurement has been reported to be useful for diagnosing gastroesophageal reflux disease (GERD). This study aimed to clarify the usefulness of salivary pepsin measurement in patients with proton pump inhibitor (PPI)-refractory GERD symptoms without erosive esophagitis.

Methods One hundred and two patients were included. Over seven days after terminating PPI treatment, all patients underwent a 24-h pH-impedance test and salivary pepsin measurement. In patients whose main symptoms included laryngopharyngeal symptoms, a hypopharyngeal multichannel intraluminal impedance (HMII) test was performed, whereas in other patients, a conventional combined multichannel intraluminal impedance-pH (MII-pH) test was performed. In the HMII tests, patients were divided into abnormal proximal exposure (APE) and non-APE groups. Salivary pepsin concentrations were compared according to acid exposure time (AET) values and were also compared between the APE and non-APE groups.

Results The median salivary pepsin concentration in patients with AET > 6% was significantly higher than that in patients with AET ≤ 6% (345.0 [170.0–469.3] ng/mL vs. 120.0 [97.0–290.1] ng/mL, $p < 0.01$). The sensitivity, specificity, positive predictive value, and negative predictive value of a positive test (> 109 ng/mL) to diagnose patients with AET > 6% were 75.0%, 51.3%, 32.1%, and 86.9%, respectively. There was no significant difference between concentrations in the APE group and concentrations in the non-APE group.

Conclusions In patients with PPI-refractory nonerosive reflux disease, salivary pepsin measurement may help diagnose patients who have conclusive evidence of reflux, whereas it is not adequate for identifying patients with APE.

Keywords GERD · LPR · Salivary pepsin · Ppi-refractory GERD · NERD

Abbreviations

GERD	Gastroesophageal reflux disease
NERD	Nonerosive reflux disease
FH	Functional heartburn
RH	Reflux hypersensitivity
AET	Acid exposure time
MII-pH	Combined multichannel intraluminal impedance-pH
HMII	Hypopharyngeal multichannel intraluminal impedance
PPI	Proton pump inhibitor
LPR	Laryngopharyngeal reflux
APE	Abnormal proximal exposure
FCR	Full column reflux

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Published online: 25 January 2020

Introduction

Gastroesophageal reflux disease (GERD) is a condition, wherein the gastric contents reflux into the esophagus, causing troublesome symptoms or complications [1]. There are a large number of GERD patients worldwide, and the number is increasing not only in Western countries but also in Asia [2, 3]. GERD is diagnosed by endoscopic examination, but reports indicate that the sensitivity and specificity of making a GERD diagnosis based only on endoscopic findings are low and that approximately 70% of patients with GERD symptoms exhibit no macroscopic evidence of esophageal mucosal injuries [4]. Therefore, the esophageal combined multichannel intraluminal impedance-pH (MII-pH) test has been used to objectively evaluate GERD [5–8]. The MII-pH test can measure both acid and non-acid reflux events and the composites of the refluxates (gas, liquid, and mixture of gas and liquid) in addition to symptom correlation. However, the current MII-pH test is a catheter-based 24-h test and causes considerable patient discomfort. Hence, methods to easily discriminate between GERD and non-GERD are wanted.

According to several guidelines, proton pump inhibitors (PPIs) are recommended as first-line therapy for GERD and are effective in 80–90% of patients with erosive esophagitis [9]. Conversely, PPIs improve symptoms in only 40–50% of patients with nonerosive reflux disease (NERD) [10]. In addition, approximately half of PPI-refractory NERD patients are reported to have functional heartburn [11, 12]. Functional heartburn is diagnosed by the absence of abnormal esophageal acid exposure and no association between symptoms and gastroesophageal reflux [13]. For treatment of functional heartburn, esophageal pain modulators, such as low-dose tricyclic antidepressants and serotonin reuptake inhibitors are recommended and is completely different from GERD treatment [13]. Therefore, documentation of abnormal esophageal acid exposure in PPI-refractory NERD patients is extremely important for determining subsequent treatment.

Recently, measurement of pepsin in saliva, which is a noninvasive and low-cost option, has been reported as a new method for GERD diagnosis [14–17]. In a previous report, a positive test had 78.6% sensitivity and 64.9% specificity for the diagnosis of GERD and reflux hypersensitivity [15]. However, no study targeting NERD patients with PPI-refractory reflux symptoms has been done.

The objective of this study is to clarify the usefulness of salivary pepsin measurement in patients with PPI-refractory GERD symptoms who did not have erosive esophagitis.

Materials and methods

Study design and participants

This was a prospective study conducted at Chiba University Hospital (Japan) involving patients with PPI-refractory GERD symptoms without any endoscopic evidence of mucosal injury, such as esophagitis or Barrett's esophagus. GERD symptoms included not only typical reflux symptoms (heartburn or regurgitation) but also laryngopharyngeal reflux (LPR) extraesophageal symptoms [1]. LPR symptoms were defined as sore throat, hoarseness, globus sensation, chronic cough, dysphonia, halitosis, difficulty breathing, regurgitation, and abnormal sensation in the laryngopharynx. Patients without reflux symptoms were excluded in advance. PPI-refractory symptoms were defined as those present when patients had not responded sufficiently to at least 8 weeks of treatment with a PPI at the usual dose (vonoprazan 20 mg, esomeprazole 20 mg, or rabeprazole 10 mg). Symptom severity was objectively assessed using the frequency scale for the symptoms of GERD (FSSG) questionnaire [18]. All patients underwent endoscopy to exclude any mucosal injury, such as esophagitis after PPI treatment. Over seven days after discontinuation of PPI treatment, all patients with no endoscopic findings of esophagitis underwent an impedance-pH test and salivary pepsin measurement. To accurately diagnose LPR, in patients whose main symptoms included LPR symptoms, a hypopharyngeal multichannel intraluminal impedance (HMII) test was performed [19–21], whereas

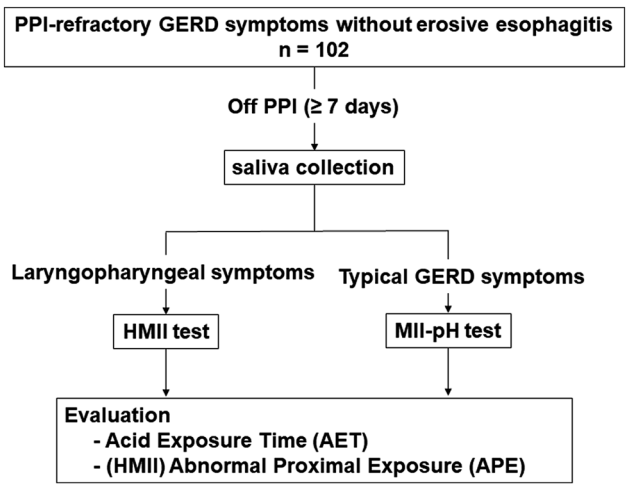

Fig. 1 Study flowchart: flowchart of patient enrollment. *GERD* gastroesophageal reflux disease, *MII-pH* multichannel intraluminal impedance-pH test, *HMII* hypopharyngeal multichannel intraluminal impedance

in patients without LPR symptoms, a conventional MII-pH test was performed (Fig. 1) [5–8].

This study was reviewed and approved by the Institutional Review Board of Chiba University School of Medicine and was registered at the University Hospital Medical Information Network (UMIN000028337). Informed consent was obtained from all participants.

Salivary pepsin measurement

Pepsin concentrations in saliva were measured using a lateral flow device (Peptest™, RD Biomed Ltd., Hull, UK). We collected samples three times a day: (1) at waking, (2) 1 h after the evening meal, and (3) just before impedance-pH testing with fasting. Just prior to impedance-pH testing, saliva samples were collected at our hospital, and 1 mL of saliva was added to 0.5 mL of 0.01 M citric acid and stored at 4 °C until measurement. Saliva samples were also collected at home at the time of waking and 1 h after the evening meal. These samples were mixed in tubes with 0.5 mL of 0.01 M citric acid. The tubes were brought to our hospital on the next day following collection. The samples were then stored at 4 °C until measurement. Pepsin concentrations in saliva were measured within seven days of sample collection.

Combined multichannel intraluminal impedance-pH (MII-pH) testing

After an overnight fast and saliva sample collection, the MII-pH catheter (Sandhill Scientific Inc., Highland Ranch, CO, USA) was inserted for 24 h. It was connected to a ZepHr reflux recording system (Sandhill Scientific Inc.) to capture pH (distal esophagus and stomach), impedance, and symptom data. The patients returned to the hospital on the subsequent day for data analysis. The MII-pH data were manually analyzed by two experienced investigators (TM and HI) using the BioView™ analysis software (Sandhill Scientific Inc.). In this study, functional heartburn was diagnosed when the acid exposure time (AET) was normal, and low symptom index (< 50%). Hypersensitive esophagus was diagnosed when the AET was normal, but the symptom index was positive (≥ 50%). Normal AET in the distal esophagus was defined as < 4% per day [8, 22], and AET > 6% was defined as conclusive evidence of pathologic reflux according to the Lyon consensus [22].

Hypopharyngeal multichannel intraluminal impedance (HMII)

A specialized catheter for HMII (CAZI-BL-55; Sandhill Scientific Inc., Highlands Ranch, Colorado, USA) that has two pairs of impedance electrodes in each hypopharynx, proximal esophagus, and distal esophagus and two pH probes (hypopharynx and distal esophagus), was used for HMII. The catheter was placed after salivary collection and was connected to a ZepHr reflux recording system (Sandhill Scientific Inc.) [19–21]. The HMII catheter was placed under laryngoscopic guidance by an otolaryngologist so that the top electrode in the hypopharynx was placed 0.5 cm proximal to the upper border of the upper esophageal sphincter. The HMII data were manually analyzed by two experienced investigators (TH and TS). An LPR event was defined as a retrograde bolus transit that crossed all catheter ring sets and reached the hypopharynx. Full column reflux (FCR) was defined as reflux that reached the impedance site 2 cm distal to the upper esophageal sphincter but did not reach the hypopharyngeal ring set. Abnormal proximal exposure (APE) was defined as LPR ≥ 1/day and/or FCR (reflux 2 cm distal to the upper esophageal sphincter) ≥ 5/day [19–21]. On the basis of the HMII measurements, the patients were divided into APE and non-APE groups.

Salivary pepsin concentrations were compared between sets of AET values (AET ≥ 4% and AET < 4%, AET ≥ 5% and AET < 5%, and AET > 6% and AET ≤ 6%) and between the APE and non-APE groups. The AET values were divided according to the Lyon consensus [22]. We also determined the sensitivity and specificity of pepsin concentrations at all possible cutoff points to predict GERD using receiver operating characteristic curves.

Sample size

The sample sizes for the patients with increased AET and those without were determined using preliminary data to fit Mann–Whitney *U* test, where maximal salivary pepsin concentration was the outcome of interest. In this preliminary data, the mean salivary pepsin concentration was 403.6 and 272.8 in patients with increased AET ($n = 5$) and those without ($n = 5$), respectively, and the response within each subject group was normally distributed with a standard deviation (SD) of 206.3. If the true difference in the experimental and control means is equal to 130.8, the study would require a sample of 88 patients (44 patients in each group) in order to be able to reject the null hypothesis (the population means of the experimental and control groups were equal) with a probability of 80% and at a significance level of 0.05. Assuming a drop rate, we thus aimed to enroll 100 patients with PPI-refractory GERD symptoms without erosive esophagitis.

Statistical analysis

Baseline data are presented as mean ± standard deviation, and median and interquartile range. Differences in clinical parameter values between the groups were analyzed using the Student's *t* test, the Chi-square test and the

Mann–Whitney *U* test. Differences in pepsin concentration between groups were analyzed using the Mann–Whitney *U* test. Receiver operating characteristic curves were used to determine the sensitivity and the specificity of pepsin concentrations at all possible cutoff points to predict GERD. All statistical analyses were performed using SPSS 23 (SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was considered statistically significant.

Results

Patients

Between June 2016 and December 2018, 102 patients with PPI-refractory GERD symptoms were enrolled in this study after endoscopic findings showed no evidence of mucosal injury, such as esophagitis or Barrett's esophagus. The clinical characteristics of the patients are shown in Table 1. Of 102 patients, 84 underwent HMII testing, and 18 underwent MII-pH testing. On the basis of the HMII results, APE was positive in 57 patients (LPR ≥ 1 with FCR ≥ 5 in 29 patients, LPR ≥ 1 without FCR ≥ 5 in 2 patients, and FCR ≥ 5 without LPR in 26 patients). Twenty-nine (50.8%) patients with APE had increased AET. Four patients with negative APE had increased AET. On the basis of the MII-pH results, five patients had increased AET. Taken together, 38 patients had increased AET (AET ≥ 4% in all 38 patients, AET ≥ 5% in 31 patients, and AET > 6% in 24 patients). There were no significant differences in patient demographics (sex, age, body mass index, and symptom severity) between patients with increased AET and those with normal AET (Table 1).

Salivary pepsin concentration and timing of saliva collection

Median pepsin concentrations at three collection times, median pepsin concentration of the maximal salivary pepsin concentrations (out of the three samples), and differences in pepsin concentrations by AET values are shown in Table 2. The maximal salivary pepsin concentrations in patients with AET ≥ 4% were significantly higher than those in patients with AET < 4% (Fig. 2). However, there were no significant differences when the results for the two groups were compared at the three collection times. By contrast, there were significant differences in pepsin concentrations at 1 h after the evening meal between patients with AET > 5% and patients with AET ≤ 5%. Furthermore, by comparing results between patients with AET > 6% and those with AET ≤ 6%, a significant difference was confirmed at waking as well as at 1 h after the evening meal. In all comparisons, there were no significant differences in results for the fasting before pH test collection time.

Median pepsin concentrations at the three collection times, median pepsin concentration of the maximal salivary pepsin concentrations, and differences between the APE and non-APE groups are shown in Table 3. There were no significant differences between the APE and non-APE groups in terms of concentrations at the three collection times and maximal concentrations.

Salivary pepsin concentration to differentiate patients with increased AET from patients without

Receiver operating characteristic analysis of salivary pepsin measurements for diagnosing AET ≥ 4% in patients with PPI-refractory GERD symptoms using a maximal pepsin concentration threshold of 187.0 ng/mL revealed 50.0%

Table 1 Participant characteristics

	Total	Patients with increased AET (AET ≥ 4%)	Patients with normal AET (AET < 4%)	*p* value*
	n = 102	*n* = 38	*n* = 64	
Sex (male/female)	65/37	28/10	37/27	0.10
Age (years, ± SD)	50.3 ± 16.6	49.9 ± 14.1	52.4 ± 17.7	0.43
BMI (kg/m^2)	22.3 ± 4.3	23.1 ± 3.6	21.7 ± 4.5	0.12
FSSG total	23.3 ± 10.6	25.1 ± 8.9	22.0 ± 11.5	0.18
FSSG reflux	13.8 ± 6.5	15.1 ± 5.4	13.0 ± 7.1	0.15
FSSG dysphagia	9.4 ± 5.2	9.6 ± 4.2	9.2 ± 5.7	0.69
AET (%)	2.4 [0.6–5.7]	6.8 [5.1–12.9]	0.9 [0.2–2.2]	$p < 0.01$†
DeMeester score	9.1 [3.1–20.2]	23.7 [17.5–36.0]	4.2 [1.7–8.3]	$p < 0.01$†

AET acid exposure time, *BMI* body mass index, *FSSG* frequency scale for the symptoms of gastroesophageal reflux disease, *n.s.* not significant

*Student's *t* test, Chi-square test, and Mann–Whitney *U* test analysis between the patients with increased AET (AET > 4%) and those with normal AET groups

†Mann–Whitney *U* test, $p < 0.05$

Table 2 Pepsin concentrations at three collection times, maximal salivary pepsin concentrations and differences in pepsin concentrations by AET values

Timing of saliva collection	AET ≥ 4% *n* = 38	AET < 4% n = 64	*p* value
Waking	36.0 [29.7–58.0]	32.5 [25.0–46.8]	0.23
Fasting before pH test	147.0 [84.0–358.0]	79.5 [33.3–246.8]	0.05
1 h after evening meal	92.0 [41.0–380.0]	42.0 [30.0–205.0]	0.10
Maximal pepsin concentration	190.0 [47.8–469.3]	108.0 [41.3–257.3]	0.04*
	AET > 5% *n* = 30	AET ≤ 5% *n* = 72	
Waking	38.0 [34.3–84.0]	14.5 [0.0–29.0]	0.05
Fasting before pH test	147.0 [72.5–344.0]	234.0 [110.0–358.0]	0.12
1 h after evening meal	107.0 [47.5–418.5]	27.0 [25.0–29.0]	0.008*
Maximal pepsin concentration	257.0 [120.0–457.0]	234.0 [110.0–358.0]	0.04*
	AET > 6% *n* = 23	AET ≤ 6% *n* = 79	
Waking	40.0 [35.5–145.5]	29.0 [0.0–34.0]	0.02*
Fasting before pH test	147.0 [84.0–358.0]	79.5 [33.3–246.8]	0.12
1 h after evening meal	246.0 [62.0–467.0]	37.0 [27.0–77.5]	0.004*
Maximal pepsin concentration	345.0 [170.0–469.3]	120.0 [97.0–290.1]	0.01*

AET acid exposure time

*Mann–Whitney *U* test, $p < 0.05$

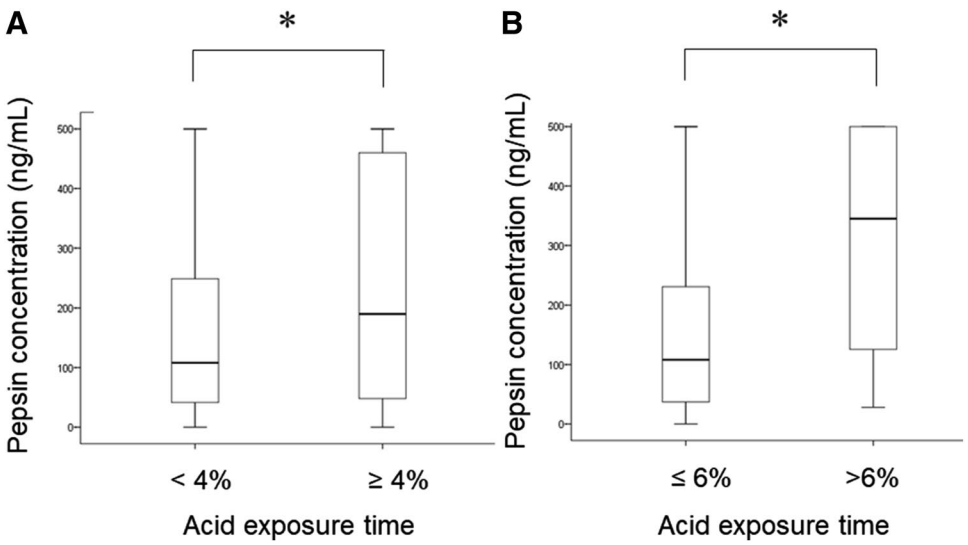

Fig. 2 Concentration of pepsin in saliva in patients with AET ≥ 4% and those with AET < 4%, and AET ≤ 6% and those with AET > 6%. **a** The maximal salivary pepsin concentration in patients with AET ≥ 4% was significantly higher than that in patients with AET < 4%. **b** The maximal salivary pepsin concentration in patients with AET > 6% was also significantly higher than that in patients with AET ≤ 6%. AET, acid exposure time

Table 3 Pepsin concentrations at three collection times, maximal salivary pepsin concentrations and differences in pepsin concentrations between the APE and non-APE groups

Timing of saliva collection	APE *n* = 57	non-APE *n* = 27	*p* value
Waking	36.0 [29.0–81.0]	33.5 [6.25–153.2]	0.73
Fasting before pH test	110.0 [54.0–300.0]	74.0 [34.5–231.7]	0.30
1 h after evening meal	48.0 [34.0–160.0]	58.0 [35.2–188.2]	0.91
Maximal pepsin concentration	194.0 [47.0–405.0]	92.0 [36.0–266.0]	0.77

Mann–Whitney *U* test

APE Abnormal proximal exposure

sensitivity, 63.5% specificity, 44.2% positive predictive value, 67.8% negative predictive value, positive likelihood ratio of 1.37, and negative likelihood ratio of 0.79 (Fig. 3a). Receiver operating characteristic analysis of salivary pepsin measurements for diagnosing AET > 6% using a maximal pepsin concentration threshold of 109.0 ng/mL revealed 75.0% sensitivity, 51.3% specificity, 32.1% positive predictive value, 86.9% negative predictive value, positive likelihood ratio of 1.54, and negative likelihood ratio of 0.43 (Fig. 3b).

Discussion

In this prospective comparative study, salivary pepsin measurement may help diagnose patients who have conclusive evidence of reflux (AET > 6%), whereas it is not adequate for identifying patients with APE in NERD patients with PPI-refractory reflux symptoms. This study is the first report of salivary pepsin measurements in PPI-refractory NERD patients.

Pepsin is a proteolytic enzyme and is thought to play a role in the epithelial damage related to GERD or LPR. Data from previously published studies describing the relationship between salivary pepsin concentrations and GERD or LPR are summarized in Table 4. In addition to the sample size, there were differences in the timing of saliva sampling, the method used to measure the pepsin concentrations, and the method used for the diagnosis of GERD or LPR among the studies. Salivary pepsin measurement has been reported as a promising diagnostic tool for GERD or LPR in some studies [14–17, 23–27], but other studies reported that salivary pepsin measurement was not useful [28–31]. In the present study, although the salivary pepsin concentration was significantly higher in patients with increased AET than in those without, the sensitivity and specificity of the positive test to diagnose AET ≥ 4% were low. However, the negative predictive value of the positive test for diagnosing AET > 6% was relatively high at 86.9% positive likelihood ratio. This result indicates that salivary pepsin measurements may be beneficial for the exclusion diagnosis of AET > 6%.

In previous studies, AET ≥ 4% or 4.2% was defined as GERD [14, 15, 26, 30], and there is no study comparing salivary pepsin concentration and AET degree. Considering the results of this study, the proportion of patients with high AET values included in previous studies may affect those studies conclusions on the usefulness of salivary pepsin measurement for GERD diagnosis. Furthermore, differences in methods other than the MII-pH test for the diagnosis of GERD may influence their conclusions. Yadolapati et al. reported that salivary pepsin measurement was not useful in diagnosing LPR in their prospective study [28]. However, the diagnosis of LPR in that study was made using oropharyngeal pH monitoring, which was different from the HMII testing modality used in the present study. Yuksel et al. reported that oropharyngeal pH monitoring appeared to be more sensitive than traditional pH monitoring in the evaluation of patients with extraesophageal reflux

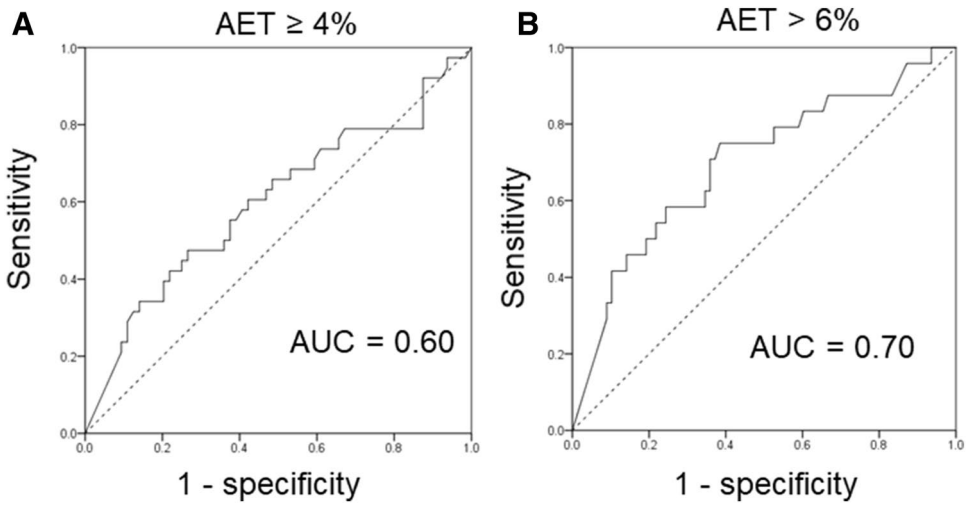

Fig. 3 Receiver operating characteristic analysis of salivary pepsin measurements using maximal pepsin concentration: **a** receiver operating characteristic analysis of salivary pepsin measurement at a threshold of 187.0 ng/mL for diagnosing patients with AET ≥ 4% in patients with PPI-refractory GERD symptoms revealed 50.0% sensitivity, 63.5% specificity, 44.2% positive predictive value, 67.8% negative predictive value, positive likelihood ratio of 1.37, and negative likelihood ratio of 0.79. **b** Receiver operating characteristic analysis of salivary pepsin measurement at a threshold of 109.0 ng/mL for diagnosing patients with AET > 6% in patients with PPI-refractory GERD symptoms revealed 75.0% sensitivity, 51.3% specificity, 32.1% positive predictive value, 86.9% negative predictive value, positive likelihood ratio of 1.54, and negative likelihood ratio of 0.43

Table 4 Main previous papers describing the relationship between pepsin concentration in saliva and GERD or LPR

Authors	Sample size and composition	Timing of saliva sample collection	Device (pepsin, pH)	Outcome
Yuksel et al. [14]	Adult, 109 (HC, 51; GERD, 58)	Not listed	PEPTEST, wireless 48-h pH monitoring	Useful
Hayat et al. [15]	Adult, 211 (HC, 100; Patients with heartburn, 111)	Three times (at waking, 1 h after lunch, and 1 h after dinner)	PEPTEST, MII-pH	Useful
Ocak et al. [17]	Adult, 20 (suspicion of LPR)	Symptomatic conditions	PEPTEST, MII-pH (double channeled)	Useful
Sereg-Bahar et al. [23]	Adult, 76 (HC, 48; LPR, 28)	2 h after a meal	Enzyme immune test, 24-h pH monitoring (Siemens Healthcare, Germany)	Useful
Yadlapati et al. [28]	Adult, 31 (HC,18; Laryngeal, 13)	Not listed	PEPTEST, Oropharygeal pH probe	Not useful
Na et al. [24]	Adult, 72 (HC,12; LPR, 50)	Four times (at waking, 1 h after each meal)	Enzyme-linked immunosorbent assay, HMII (LPR catheter: ZAI-BL-54)	Useful
Dy et al. [29]	Children, 50	Fasting before MII-pH test	PEPTEST, MII-pH	Not useful
Fortunato et al. [25]	Children, 133 (symptomatic, 90; asymptomatic, 43)	Eight times (at waking, before MII-pH, and before and after meals)	Enzyme-linked immunosorbent assay, MII-pH	Useful
Hashizume et al. [30]	Children (severe and intellectual disabilities, 26; GERD, 15; non-GERD, 11)	Two times (morning fasting and post-enteral feeding)	PEPTEST, MII-pH	Not useful
Du et al. [26]	Adult, 285 (patients with GERD symptoms, 250; HC, 35)	Three times (at waking, 1 h after lunch, and 1 h after dinner)	PEPTEST, MII-pH	Useful
Li et al. [27]	Adult, 56 (patients with GERD symptoms, 45; HC, 11)	Three times (at waking, symptomatic condition, and before bedtime)	Enzyme-linked immunosorbent assay, MII-pH	Useful
Woodland et al. [31]	Adult, 61 (patients with heartburn, 44; HC, 17)	Three times (at waking, 1 h after lunch, and 1 h after dinner)	PEPTEST, MII-pH	Not useful

GERD gastroesophageal reflux disease, *LPR* laryngopharyngeal reflux disease, *HC* healthy control, *MII-pH* multichannel intraluminal impedance test combined with a pH, *HMII* hypopharyngeal multichannel intraluminal impedance

[32]. Although some studies suggested the benefit of oropharyngeal pH monitoring, it was recently reported that the correlation between oropharyngeal pH-metry and MII-pH was weak [33]. In addition, Yadlapati et al. reported that oropharyngeal pH monitoring did not distinguish between healthy volunteers and subjects with both laryngeal and reflux symptoms [28]. Taken together, the "true" benefit of oropharyngeal pH monitoring remains unclear.

We believed that the accurate diagnosis of LPR was crucial for this study. Since a conventional MII-pH test cannot detect actual LPR events, we used HMII to directly measure LPR in patients with LPR symptoms. The potential benefit of using HMII to diagnose LPR and to select patients for anti-reflux surgery has been reported. Hoppo and colleagues have reported that 70% of patients with "adult-onset" asthma and chronic cough had APE based on HMII and had significant symptomatic improvement (90% of patients with adult-onset asthma) or complete symptom resolution (81% of patients with chronic cough) after anti-reflux surgery [20, 21]. Most recently, Suzuki et al. reported that 100% of Japanese patients with LPR symptoms or laryngoscopic findings suggestive of LPR who had APE on HMII had significant symptomatic improvement with anti-reflux surgery [34]. These data suggest that APE as measured by HMII could be a good indicator for selecting patients with LPR symptoms who will likely respond to anti-reflux surgery. In the present study, there was no significant difference between measurements in the APE and non-APE groups. Our data are supported by the work of Komatsu et al., who used Western blot analysis to measure the protein levels of pepsin in hypopharyngeal biopsy specimens from 17 patients with APE and three patients without APE. They demonstrated that there was no significant difference in pepsin protein levels between the APE and non-APE groups [35]. Since it has been reported that APE contained several non-acid refluxes [34], we thought that there would be no difference in salivary pepsin concentration between the APE and non-APE groups. Since as few as three LPR events a week could cause severe laryngeal inflammation [36], 24-h monitoring may miss the actual LPR events. In addition to HMII, other markers are needed to identify patients with "true" LPR events that would benefit from anti-reflux surgery.

In the present study, we collected saliva samples at waking, 1 h after the evening meal, and after fasting just before

the pH test. As a result, there were differences between patients with AET > 6% and those with AET ≤ 6% at waking and at 1 h after the evening meal. However, there were no significant differences after fasting just before the pH test. Several reports have described the timing of the saliva collection (Table 4), but there has been no general consensus yet. Hayat et al. reported that sampling during the postprandial period (1 h after finishing lunch or dinner) was optimal for GERD diagnosis [15]. Na et al. reported that collection of saliva at waking was preferable for LPR diagnosis [24]. Fortunato et al. reported that a saliva sample must be obtained soon after a reflux event [25]. As we did in the present study, Hashizume et al. measured salivary pepsin early in the morning with fasting before pH-impedance testing in 26 patients with severe motor and intellectual disabilities [30]. From the results of this study, although we did not collect saliva soon after the reflux symptoms, we determined that the most suitable time for collection was 1 h after evening meal among the three points we measured.

Regarding the usefulness of salivary pepsin measurement for NERD patients, Li et al. reported that pepsin concentration was significantly higher in 10 patients with NERD than that in 10 patients with functional heartburn [27]. However, their subjects were not PPI-refractory NERD patients. Appropriate management of PPI-refractory NERD patients is known to be difficult in clinical practice. Therefore, we believe that our study, which showed that salivary pepsin measurement may be useful in identifying patients with severe acid reflux in PPI-refractory NERD patients, is clinically important.

Our study has several limitations. First, we examined only patients without erosive esophagitis; therefore, it appears unclear whether the salivary pepsin measurement is generally inadequate for all patients with APE. Second, all the patients underwent endoscopy after PPI treatment to exclude erosive esophagitis. Therefore, patients who had erosive esophagitis when they underwent endoscopy before PPI treatment or after cessation of PPI treatment may have been included.

In conclusion, we demonstrated the potential benefit of salivary pepsin measurements as a promising clinical diagnostic modality for NERD patients with PPI-refractory GERD symptoms. This simple, low-cost testing may be useful to evaluate NERD patients with PPI-refractory GERD symptoms and identify patients who have conclusive evidence of reflux.

Acknowledgements We thank Atsuko Kikuchi and Mai Fujie for their help on 24-h pH impedance test.

Author contributions TM and TS designed the study, conducted the experiment, collected data, analyzed and interpreted data, and wrote the manuscript. TM, AM, TS, and TH designed the study, analyzed, and interpreted data, assisted in writing the manuscript. YS, SK, HI, KI, KO, NA, DM, and TN assisted in conducting the experiment and collecting data. TO, YO, and NK assisted in interpreting data and writing the manuscript. All authors approved the final version of the manuscript.

Funding This work was supported by JSPS KAKENHI Grant No. 18K09367.

Compliance with ethical standards

Ethical Statement All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study.

Conflict of interest The authors indicated no potential conflicts of interest.

References

1. Vakil N, van Zanten SV, Kahrilas P, et al. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol. 2006;101:1900–20.
2. Dent J, El-Serag HB, Wallander MA, et al. Epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut. 2005;54:710–7.
3. Fujiwara Y, Arakawa T. Epidemiology and clinical characteristics of GERD in the Japanese population. J Gastroenterol. 2009;44:518–34.
4. Lundell LR, Dent J, Bennett JR, et al. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. Gut. 1999;45:172–80.
5. Kahrilas PJ, Shaheen NJ, Vaezi MF, American Gastroenterological Association Institute; Clinical Practice, and Quality Management Committee. American Gastroenterological Association Institute technical review on the management of gastroesophageal reflux disease. Gastroenterology. 2008;135:1392–413.
6. Vaezi MF. Role of impedance/pH monitoring in refractory gerd: let's be careful out there! Gastroenterology. 2007;132:1621–16212.
7. Lacy BE, Weiser K, Chertoff J, et al. The diagnosis of gastroesophageal reflux disease. Am J Med. 2010;123:583–92.
8. Kahrilas PJ, Quigley EM. Clinical esophageal pH recording: a technical review for practice guideline development. Gastroenterology. 1996;110:1982–96.
9. Dekkers CP, Beker JA, Thjodleifsson B, et al. Double-blind comparison [correction of Double-blind, placebo-controlled comparison] of rabeprazole 20 mg vs. omeprazole 20 mg in the treatment of erosive or ulcerative gastro-oesophageal reflux disease. The European Rabeprazole Study Group. Aliment Pharmacol Ther. 1999;13:49–57.
10. Fass R. Erosive esophagitis and nonerosive reflux disease (NERD): comparison of epidemiologic, physiologic, and therapeutic characteristics. J Clin Gastroenterol. 2007;41:131–7 **(Review)**.
11. Mainie I, Tutuian R, Shay S, et al. Acid and non-acid reflux in patients with persistent symptoms despite acid suppressive therapy: a multicentre study using combined ambulatory impedance-pH monitoring. Gut. 2006;55:1398–402.
12. Matsumura T, Ishigami H, Fujie M, et al. Endoscopic-guided measurement of mucosal admittance can discriminate

gastroesophageal reflux disease from functional heartburn. Clin Transl Gastroenterol. 2017;8:e94.
13. Aziz Q, Fass R, Gyawali CP, et al. Esophageal disorders. Gastroenterology. 2016;150:1368–79.
14. Saritas Yuksel E, Hong SK, Strugala V, et al. Rapid salivary pepsin test: blinded assessment of test performance in gastroesophageal reflux disease. Laryngoscope. 2012;122:1312–6.
15. Hayat JO, Gabieta-Somnez S, Yazaki E, et al. Pepsin in saliva for the diagnosis of gastro-oesophageal reflux disease. Gut. 2015;64:373–80.
16. Hayat JO, Yazaki E, Moore AT, et al. Objective detection of esophagopharyngeal reflux in patients with hoarseness and endoscopic signs of laryngeal inflammation. J Clin Gastroenterol. 2014;48:318–27.
17. Ocak E, Kubat G, Yorulmaz İ. Immunoserologic pepsin detection in the saliva as a non-invasive rapid diagnostic test for laryngopharyngeal reflux. Balkan Med J. 2015;32:46–50.
18. Kusano M, Shimoyama Y, Sugimoto S, et al. Development and evaluation of FSSG: frequency scale for the symptoms of GERD. J Gastroenterol. 2004;39:888–91.
19. Hoppo T, Jarido V, Pennathur A, et al. Antireflux surgery preserves lung function in patients with gastroesophageal reflux disease and end-stage lung disease before and after lung transplantation. Arch Surg. 2011;146:1041–7.
20. Komatsu Y, Hoppo T, Jobe BA. Proximal reflux as a cause of adult-onset asthma: the case for hypopharyngeal impedance testing to improve the sensitivity of diagnosis. JAMA Surg. 2013;148:50–8.
21. Hoppo T, Komatsu Y, Jobe BA. Antireflux surgery in patients with chronic cough and abnormal proximal exposure as measured by hypopharyngeal multichannel intraluminal impedance. JAMA Surg. 2013;148:608–15.
22. Gyawali CP, Kahrilas PJ, Savarino E, et al. Modern diagnosis of GERD: the Lyon consensus. Gut. 2018;67:1351–62.
23. Sereg-Bahar M, Jerin A, Jansa R, et al. Pepsin and bile acids in saliva in patients with laryngopharyngeal reflux—a prospective comparative study. Clin Otolaryngol. 2015;40:234–9.
24. Na SY, Kwon OE, Lee YC, et al. Optimal timing of saliva collection to detect pepsin in patients with laryngopharyngeal reflux. Laryngoscope. 2016;126:2770–3.
25. Fortunato JE, D'Agostino RB Jr, Lively MO. Pepsin in saliva as a biomarker for oropharyngeal reflux compared with 24-hour esophageal impedance/pH monitoring in pediatric patients. Neurogastroenterol Motil. 2017;29:e12936.
26. Du X, Wang F, Hu Z, et al. The diagnostic value of pepsin detection in saliva for gastro-esophageal reflux disease: a preliminary study from China. BMC Gastroenterol. 2017;17(17):107.
27. Li YW, Sifrim D, Xie C, Chen M, Xiao YL. Relationship between salivary pepsin concentration and esophageal mucosal integrity in patients with gastroesophageal reflux disease. J Neurogastroenterol Motil. 2017;30(23):517–25.
28. Yadlapati R, Adkins C, Jaiyeola DM, et al. Abilities of oropharyngeal pH tests and salivary pepsin analysis to discriminate between asymptomatic volunteers and subjects with symptoms of laryngeal irritation. Clin Gastroenterol Hepatol. 2016;14:535–42.
29. Dy F, Amirault J, Mitchell PD, et al. Salivary pepsin lacks sensitivity as a diagnostic tool to evaluate extraesophageal reflux disease. J Pediatr. 2016;177:53–8.
30. Hashizume N, Fukahori S, Asagiri K, et al. The characteristics of salivary pepsin in patients with severe motor and intellectual disabilities. Brain Dev. 2017;39:703–9.
31. Woodland P, Singendonk MMJ, Ooi J, et al. Measurement of salivary pepsin to detect gastroesophageal reflux disease is not ready for clinical application. Clin Gastroenterol Hepatol. 2018;17:563–5 **[Epub ahead of print]**.
32. Yuksel ES, Slaughter JC, Mukhtar N, et al. An oropharyngeal pH monitoring device to evaluate patients with chronic laryngitis. Neurogastroenterol Motil. 2013;25:315–23.
33. Mazzoleni G, Vailati C, Lisma DG, et al. Correlation between oropharyngeal pH-monitoring and esophageal pH-impedance monitoring in patients with suspected GERD-related extra-esophageal symptoms. Neurogastroenterol Motil. 2014;26:1557–644.
34. Suzuki T, Seki Y, Okamoto Y, et al. Hypopharyngeal multichannel intraluminal impedance leads to the promising outcome of antireflux surgery in Japanese population with laryngopharyngeal reflux symptoms. Surg Endosc. 2018;32:2409–19.
35. Komatsu Y, Kelly LA, Zaidi AH, et al. Hypopharyngeal pepsin and Sep70 as diagnostic markers of laryngopharyngeal reflux: preliminary study. Surg Endosc. 2015;29:1080–7.
36. Koufman JA. The otolaryngologic manifestations of gastroesophageal reflux disease (GERD): a clinical investigation of 225 patients using ambulatory 24-hour pH monitoring and an experimental investigation of the role of acid and pepsin in the development of laryngeal injury. Laryngoscope. 1991;101((4 Pt 2 Suppl 53)):1–78.