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Original Article

Salivary Pepsin as an Intrinsic Marker for Diagnosis of Sub-types of Gastroesophageal Reflux Disease and Gastroesophageal Reflux Disease-related Disorders

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Background/Aims
To determine the value of salivary pepsin in discriminating sub-types of gastroesophageal reflux disease (GERD) and GERD-related disorders.

Methods
Overall, 322 patients with different sub-types of GERD and 45 healthy controls (HC) were studied. All patients took Gastroesophageal Reflux Disease Questionnaire (GerdQ) and underwent endoscopy and 24-hour esophageal pH monitoring and manometry. Salivary pepsin concentration (SPC) was detected by using colloidal gold double-antibody immunological sandwich assay. Oral esomeprazole treatment was administrated in the patients with non-erosive reflux disease (NERD) and extra-esophageal symptoms (EES).

Results
Compared to HC, patients with erosive esophagitis, NERD, EES, EES plus typical GERD symptoms, or Barrett's esophagus had a higher prevalence of saliva and SPC (all $P < 0.001$). There was no significant difference in the positive rate for pepsin in patients with functional heartburn or GERD with anxiety and depression, compared to HC. After esomeprazole treatment, the positive rate and SPC were significantly reduced in NERD (both $P < 0.001$) and in EES ($P = 0.001$ and $P = 0.002$, respectively). Of the 64 NERD patients, 71.9% (n = 46) were positive for salivary pepsin, which was significantly higher than the rate (43.8%, n = 28) of pathological acid reflux as detected by 24-hour esophageal pH monitoring ($P = 0.002$).

Conclusions
Salivary pepsin has an important significance for the diagnosis of GERD and GERD-related disorders. Salivary pepsin and 24-hour esophageal pH monitoring may complement with each other to improve the diagnostic efficiency.
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Key Words
Anxiety; Esophageal pH monitoring; Gastroesophageal reflux; Gold colloid; Saliva

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Introduction

Gastroesophageal reflux disease (GERD) is "a condition that develops when the reflux of stomach contents into the esophagus and causes troublesome symptoms and/or complications," the prevalence of GERD has increased in recent years, with significant impact on the quality of life.[1-3] Heartburn and regurgitation are the typical symptoms of GERD, counting for 40-60% of entire patient community, whereas the proportion of symptoms manifesting as cough, hoarseness, asthma and pharyngitis, is 70-90% in extra-esophageal symptoms, which are often misdiagnosed as respiratory or throat diseases with poor or delayed management.[4-6] Stress and mental factors can increase the sensitivity of the esophagus, thereby worsening reflux symptoms in patients with GERD. In turn, GERD can also increase the incidence of psychological disorders such as anxiety and depression.[7,8] Current diagnostic methods for GERD include symptom questionnaires and 24-hour pH monitoring,[9] high-resolution esophageal manometry,[10] endoscopy, radionuclide gastroesophageal reflux test, and proton pump inhibitor (PPI) diagnostic treatments.[11] Among them, 24-hour pH monitoring is the most commonly used method with a high specificity. However, it is an invasive procedure, and associated with disadvantages such as poor patient tolerance, high cost, and difficulty in carrying out in primary hospitals. The above-mentioned diagnostic methods have their own advantages and limitations, so a new non-invasive method that is simple and sensitive for the diagnosis of GERD is in great need.

Pepsin, which is secreted by the chief cells and activated in acidic gastric secretions, is one of the main components in the reflux fluid and one of the major injury factors in patients with GERD. Pepsin is deactivated at pH 7.0, and reactivated after re-acidification.[12] When reflux occurs, pepsin can enter the oral cavity with the reflux fluid and be mixed into the saliva. Therefore, GERD can be predicted by the change of pepsin concentration in saliva, and thus saliva pepsin may play an important role in the diagnosis of GERD.[13,14] This detected the presence of pepsin in the saliva, sputum, alveolar lavage fluid and middle ear effusion as a sign of previous reflux events providing a basis for the diagnosis of reflux diseases.[13-16]

The Peptest kit, which contains 2 anti-human pepsin antibodies, using colloidal gold immunochromatography and antigen-antibody reaction principles to specifically capture the pepsin in the sample without influence by the pepsin activity, can be used in rapid detection of salivary pepsin concentration (SPC) by using colloidal gold immunochromatography and highly specific antigen-antibody reaction.[13,17] Research on pepsin detection for the diagnosis of GERD and GERD-related disorder is a hot spot nowadays,[13-17] but rarely reported in China. Moreover, incidence of GERD is increased and symptoms of GERD are worsened in Chongqing city by factors such as spicy diet. In this study, we aim to confirm that salivary pepsin test has a definite diagnostic value for different sub-types of GERD and GERD-related disorder, and it is expected to become an important diagnostic method for GERD and benefit more patients when combined with traditional diagnostic methods.

Materials and Methods

Subjects

From January 2015 to November 2018, 45 healthy controls (HC) (21 male, 24 female, age 42.4 ± 14.5 years) and 322 patients (159 male, 163 female, age 44.7 ± 15.9 years) were recruited in this study. The 322 patients were further grouped into erosive esophagitis (EE, n = 38), non-erosive reflux disease (NERD, n = 64), functional heartburn (FH, n = 44), Barrett's esophagus (BE, n = 40), GERD patients with anxiety and depression (GERD-AD, n = 48), extra-esophageal symptoms (EES, n = 44), and EES and typical GERD symptoms (EES + T-GERD, n = 44) (Fig. 1).

Each of the groups was defined as follows: EE was diagnosed in the presence of typical GERD symptoms (heartburn and/or regurgitation), 2 or more episodes per week, persistent or recurrent for more than 3 months, and the total Gastroesophageal Reflux Disease Questionnaire (GerdQ) score ≥ 8, plus erosive esophagitis as diagnosed by endoscopy; NERD was diagnosed in the presence of above clinical manifestation and GerdQ score ≥ 8, but without endoscopic erosive esophagitis; FH was diagnosed in the presence of heartburn symptom, but with normal endoscopy, normal 24-hour esophageal pH monitoring and manometry; BE was detected by endoscopy, plus typical GERD symptoms; GERD-AD was diagnosed in the presence of anxiety and/or depression symptoms as assessed by the Hamilton Anxiety and Depression Rating Scale, plus typical GERD symptoms; EES was assessed according to Montreal Consensus with symptoms manifesting as chronic cough, hoarseness, asthma, globus sensation, pharyngitis, reflux tooth erosion, or other respiratory symptoms for more than 3 months[1]; and EES + T-GERD was diagnosed in the presence of both EES and typical GERD symptoms. The study protocol was approved by the Ethics Committee of the Army Medical Center (Approval No. 2016-21), and the patients and HC enrolled participants provided

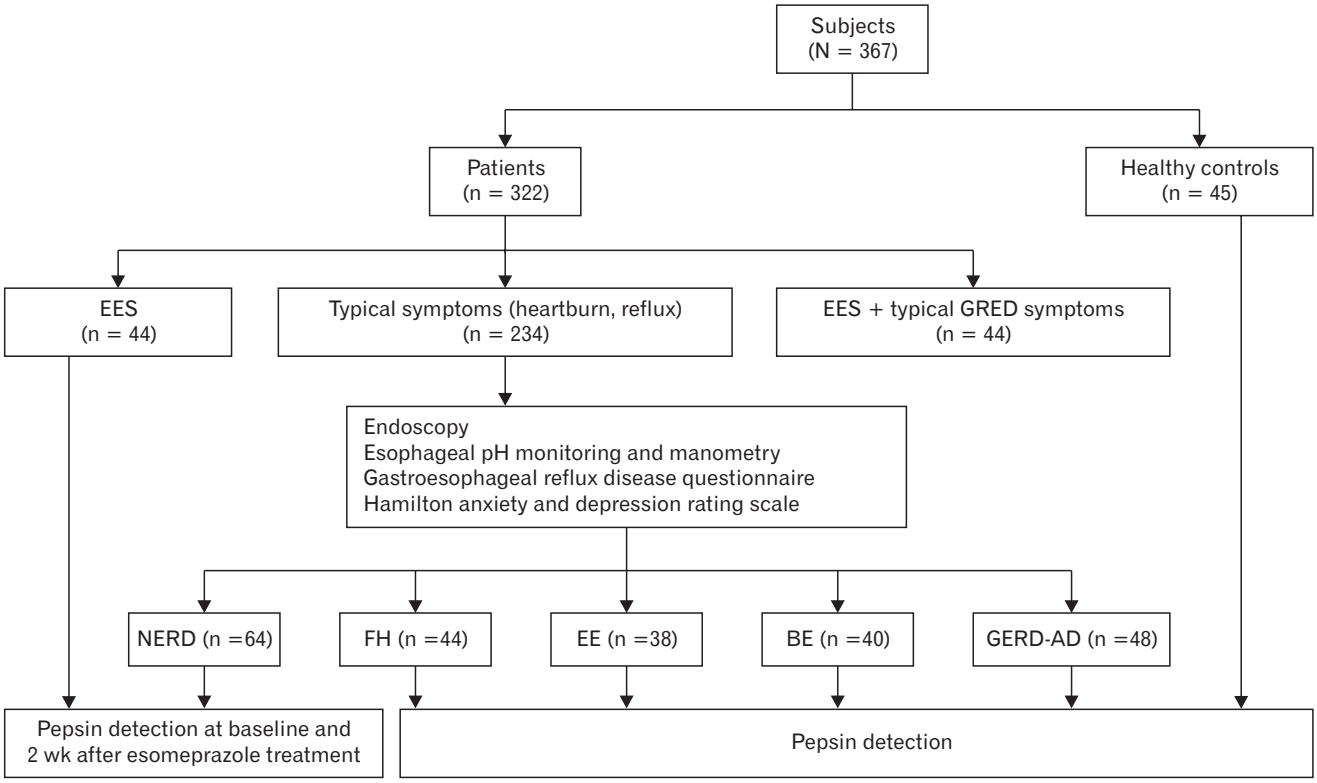

Figure 1. Flowchart of recruitment of healthy controls (HC) and patients with sub-types of gastroesophageal reflux disease (GERD) and GERD-related disorders. EES, extra-esophageal symptoms; NERD, non-erosive reflux disease; FH, functional heartburn; EE, erosive esophagitis; BE, Barrett's esophagus; GERD-AD, GERD symptoms with anxiety and depression.

written informed consent. There was no significant difference in the male to female ratio ($\chi^2 = 10.192$, $P = 0.070$), the mean age ($F = 1.402$, $P = 0.208$) among the groups.

Exclusion criteria: patients under age of 18, pregnant or lactating women, patients with achalasia, diffuse esophageal spasm, nutcracker esophagus, pharyngeal organic diseases, coronary heart disease, cancer, peptic ulcer disease, esophageal varices, and those with a history of digestive tract surgery were excluded. In addition, the patients with Zollinger-Ellison syndrome and its complications were also excluded. Patients with abnormal lung function, chest radiographs suggesting respiratory diseases, diabetes, alcoholics, or other diseases that may cause GERD-related symptoms, those drinking tea, coffee, carbonated drinks, and other foods that may affect esophageal motility in the past 2 weeks, those taking PPI (except for PPI intervention groups) and/or histamine H2 receptor antagonist (H2RA), antacids, gastric motility stimulants, and anti-acetylcholine drugs in the past 2 weeks, and those receiving acid suppression treatment or other anti-reflux treatments were also excluded.

Gastroesophageal Reflux Disease Questionnaire

The GerdQ is a 6-item tool developed to contribute to GERD diagnosis based on the symptoms found in patients, and the previous studies suggested that GerdQ may be used for diagnosis of GERD.[18,19] In a multicenter survey using GerdQ,[20] 8065 GERD patients in the Chinese population were included. Overall, 1435 (17.8%) patients had reflux esophagitis, and 620 (43.2%) of these patients had a GerdQ score of ≥ 8. Among 2025 patients with GerdQ ≥ 8, 620 (30.6%) were found to have reflux esophagitis, but the remaining 69.4% (1405/2025) had non-erosive esophagitis. Therefore, in the present study, GerdQ score was used to diagnose GERD, with the cut-off value being set as GerdQ score of ≥ 8.

Ambulatory 24-Hour pH Monitoring

Recording of the 24-hour esophageal pH monitoring was conducted with a multi-use VersaFlex catheter (Given Scientific Instruments Inc, Los Angeles, CA, USA). The pH electrode was calibrated using pH 7.0 and pH 1.0 buffer solutions before the pro-

cedure. The catheter was transnasally placed and the electrode was positioned 5 cm above the proximal border of the lower esophageal sphincter. All data were recorded using the Digitrapper equipment (Given Scientific Instruments Inc, Los Angeles, CA, USA). Abnormal esophageal acid exposure was defined as a total percentage time of greater than 4% with a pH < 4.0 and a DeMeester score > 14.7. The electrodes were fixed to the middle and lower parts of the sternum and connected to the Digitrapper pH to begin data recording. After 24 hours, the monitored data were transferred to a computer and analyzed with Accu View (Sierra Scientific Instruments, Culver City, CA, USA). During patient monitoring, the normal routine and diet were kept, with the time of eating, lying, and symptom onset being recorded. The patient was asked to avoid extra eating in addition to 3 meals a day, and try to avoid carbonated drinks, alcohol, and acidy foods; PPI/H2RA, other antacids, prokinetic agents, and non-steroidal anti-inflammatory drugs were also avoided.

Salivary Pepsin Collection and Detection

SPCs were detected by using the colloidal gold double-antibody immunological sandwich assay of the Peptest kit (RD Biomed Ltd, Hull, UK). A Peptest quantitative analyzer was used to quantitatively detect the SPC.[13] Sampling time: if there was an onset of typical symptoms of GERD, the sample was collected within 15 minutes after the onset of symptoms. If there was no onset of symptoms, or the patient could not judge by him/herself, the sample was collected 1 hour after the dinner. At least 1 mL of saliva from the throat was collected into a collection tube with 1.5 mL of citric acid. Pre-sampling precautions: caffeinated beverages, carbonated drinks, and smoking 1 hour before sampling were avoided. Taking alkaline water or beverages, antacids, and alginate antacids 48 hours before sampling, and sampling immediately after strenuous exercise were also avoided.

Detection of SPC: the Peptest kit is designed based on the colloidal gold double-antibody immunological sandwich method. It is an in vitro medical diagnostic instrument containing a reagent strip for lateral flow immunoassay. The reagent strip contains 2 monoclonal antibodies against pepsin, one is used to capture pepsin in the saliva and the other is used to detect pepsin levels in the saliva samples. The procedures are illustrated as follows: the collection tube with the sample was directly centrifuged at 4000 rpm for 5 minutes and 80 μL of supernatants was drawn with a pipette and added into a microtube with a screw cap containing 240 μL of special transfer buffer. The sample was mixed for 10 minutes on a vortex shaker. The test strip was taken out of the aluminum foil bag, and placed on a horizontal desktop, with the observation window facing upwards; The 80 μL sample was added into the injection hole on the reagent strip, and qualitative test results was read after 15 minutes. The above-mentioned colored reagent strip was placed in a special Peptest quantitative instrument to quantitatively detect the concentration of pepsin.

The salivary pepsin test is a quantitative assay that yields a specific pepsin concentration, which can be qualitatively analyzed as follows: negative with a SPC of 75 ng/mL or less, weak positive with a SPC of 75-125 ng/mL, medium positive with a SPC of 125-200 ng/mL, strong positive with a SPC of 200 ng/mL or more. Using the receiver operating characteristic (ROC) curve analysis, the optimal enthalpy SPC was determined to distinguish between the patients with GERD and those without GERD, and the value of the Youden index was greatest when the optimal pepsin value was determined to be 75 ng/mL,[13] therefore, a cut-off value of 75 ng/mL was used for the positive result of salivary pepsin test in this study.

Hamilton Anxiety and Depression Rating Scale

The Hamilton Anxiety and Depression Rating Scale[21] was used to enrolled subjects in the GERD with anxiety and depression groups. Patients were scored according to the severity from 0 to 4. Severe anxiety was defined as a total score of 29 or more; obvious anxiety was defined as a total score of 21 or more; definite anxiety was defined as a total score of 14 or more; possible anxiety was defined as a total score of 7 or more; and no anxiety symptoms was defined as a total score of 7 or less. Hamilton Depression Rating Scale: the patients were scored according to severity from 0-4. Severe depression was defined as a total score of 30 or more; moderate depression was defined as a total score of 20 or more; mild depression was defined as a total score of 17 or more; and no depression was defined as a total score of 17 or less.

Assessment of Reflux Symptom Frequency and Severity

The Reflux Disease Questionnaire[22] were used to assess the severity and frequency of GERD symptoms. The severity was categorized as score 0 (asymptomatic), 1 (mild symptoms can be tolerated), 2 (moderate, between scores 1 and 3), and 3 (severe symptoms that cannot be tolerated, and need resting). The frequency of GERD symptoms was categorized as 0 (asymptomatic), 1 (monthly), 2 (weekly), and 3 (daily).

Proton Pump Inhibitor Treatment

Oral esomeprazole magnesium was administrated (AstraZeneca Pharmaceutical Co, Ltd London, UK) at 40 mg daily continuously for NERD and EES groups for 2 weeks, and the effect of esomeprazole on salivary pepsin was compared.

Statistical Methods

Statistical analysis was performed using SPSS version 20.0 software (IBM Corp, Armonk, NY, USA). Continuous variables with normal distribution were expressed as mean ± standard deviation and those with non-normal distribution variables were reported as median (interquartile range). One-way analysis of variance, was used for the comparison of variables with normal distribution, the enumeration data was expressed as percentage or rate (%), and the Chi-square test was applied to compare the positive rates of pepsin between groups. The variable of salivary pepsin value satisfies the non-normal distribution, and thus the Mann-Whitney non-parametric test was used for comparison between groups. In addition, logistic regression was performed to evaluate to the correlation between the decrease in SPC and the improvement of GERD symptoms. A *P*-value of < 0.05 was considered statistically significant.

The following formula was used to calculate the sample size, which was based on 2 independent sample rates:

$$N = \frac{(u_\alpha + u_\beta)^2 4\pi_c(1-\pi_c)}{(\pi_1 - \pi_2)^2}$$

Note: π_1 and π_2 were the sample rates of the 2 groups, and π_c was the combined rate of the 2 groups, taking $\alpha = 0.05$, $\beta = 0.10$, then $u_\alpha = 1.96$, $u_\beta = 1.28$.

Results

Positive Rates of Pepsin in Saliva

Totally, 9/45 (20.0%) HC was positive in salivary pepsin. Compared to the HC group, the patients with EE, NERD, BE, EES, and EES + T-GERD had significantly higher positive rates of pepsin in saliva (all $P < 0.001$; Table 1). Compared with HC group, there was no significantly difference in the positive rate for pepsin in FH ($P = 0.410$) and GERD-AD ($P = 0.710$) groups. Compared with EES + T-GERD group, the positive rate of salivary pepsin was significantly increased in HC, FH, and GERD-AD groups (all $P < 0.001$). By contrast, patients with BE had a significantly higher positive rate of salivary pepsin than patients with FH ($P < 0.001$).

Pepsin Concentration in Saliva

Compared to the HC group, patients with EE, NERD, EES, EES + T-GERD and BE had significantly higher SPC (all $P < 0.001$; Fig. 2). Compared to the FH group, SPC was significantly higher in EE ($P = 0.035$) and NERD groups ($P < 0.001$; Table 2). Compared to GERD-AD group, the SPC was significantly increased in EE ($P = 0.020$) and NERD group ($P < 0.001$; Table 3).

Table 1. Positive Rates of of Pepsin in Saliva in Patients With Sub-types of Gastroesophageal Reflux Disease and Gastroesophageal Reflux Disease-related Disorders

Group	Positive rates (%)			
	> 200 ng/mL	125-200 ng/mL	75-125 ng/mL	Total positive rate
HC (n = 45)	8.9	6.7	4.4	20.0%[a]
EE (n = 38)	60.5	10.5	5.3	76.3%[b]
NERD (n = 64)	62.5	9.4	0.0	71.9%[b]
BE (n = 40)	47.5	12.5	5.0	65.0%[b,c]
EES(n = 44)	54.6	4.6	9.1	68.2%[b]
EES + T-GERD (n = 44)	64.0	4.6	4.6	72.7%[b]
GERD-AD (n = 48)	12.5	4.2	4.2	20.8%[a]
FH (n = 44)	15.9	4.6	4.6	25.0%[a]

[a]Compared to with EES + T-GERD group, $P < 0.001$.
[b]Compared to HC group, $P < 0.001$.
[c]Compared to FH group, $P < 0.001$.
HC, healthy controls; EE, erosive esophagitis; NERD, non-erosive reflux disease; BE, Barrett's esophagus; EES, extra-esophageal symptoms; EES + T-GERD, EES and typical GERD symptoms; GERD-AD, GERD patients with anxiety and depression; FH, functional heartburn.

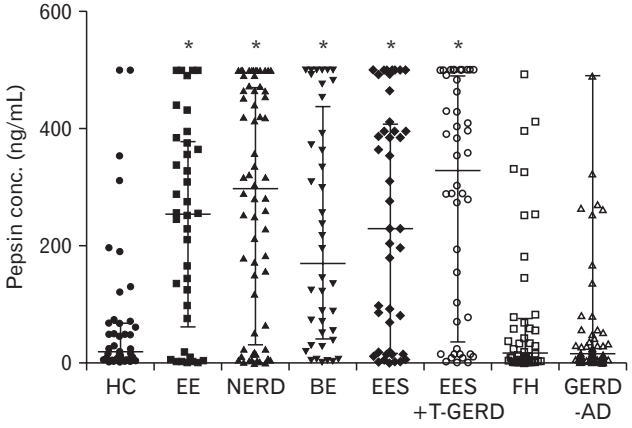

Figure 2. Scatter plot of distribution, medians and interquartile ranges (IQR; Q25, Q75) of pepsin in saliva in patients with subtypes of gastroesophageal reflux disease (GERD) and GERD-related disorders. A long horizontal line represents Median, 2 short horizontal lines represents IQR in scatter plot respectively. Compared with healthy controls (HC) group, *$P < 0.001$. EE, erosive esophagitis; NERD, non-erosive reflux disease; BE, Barrett's esophagus; EES, extra-esophageal symptoms; EES + T-GERD, EES and typical GERD symptoms; FH, functional heartburn; GERD-AD, GERD symptoms with anxiety and depression.

Table2. Comparison of Pepsin Concentration in Saliva Between Erosive Esophagitis/Non-erosive Reflux Disease Groups and Functional Heartburn

Group	Median (Q25-Q75) (ng/mL)	Z	*P*-value
FH	18.0 (8.0-74.5)		
EE	254.5 (77.0-376.0)	28.6	0.035
NERD	298.0 (39.0-468.5)	30.9	< 0.001

Q25, lower quartile; Q75, upper quartile; FH, functional heartburn; EE, erosive esophagitis; NERD, non-erosive reflux disease.

Effect of Proton Pump Inhibitor Treatment on Salivary Pepsin and Gastroesophageal Reflux Disease Symptoms in Patients With Non-esophageal Reflux Disease and Extra-esophageal Symptoms

The positive rates of pepsin in saliva and SPC were significantly decreased in the NERD group (both $P < 0.001$), and also in the EES group ($P = 0.001$ and $P = 0.002$, respectively) after PPI treatment (Table 4). Heartburn and acid regurgitation symptoms scores of GERD symptoms were both significantly decreased (4.6 ± 0.9 vs 2.4 ± 1.0, and 4.5 ± 1.1 vs 2.4 ± 0.9, respectively, both $P < 0.01$) after esomeprazole treatment, compared with those before treatment. Logistic regression analysis showed that there was a statistically significant correlation between the decrease in SPC and the improvement of GERD symptoms ($r = 0.389$, $P < 0.001$).

Table 3. Comparison of Pepsin Concentration in Saliva Between Erosive Esophagitis/Non-erosive Reflux Disease Groups and Gastroesophageal Reflux Disease With Anxiety and Depression

Group	Median (Q25-Q75) (ng/mL)	Z	*P*-value
GERD-AD	17.5 (9.0-55.5)		
EE	254.5 (77.0-376.0)	3.5	0.020
NERD	298.0 (39.0-468.5)	4.8	< 0.001

Q25, lower quartile; Q75, upper quartile; GERD-AD, gastroesophageal reflux disease with anxiety and depression; EE, erosive esophagitis; NERD, non-erosive reflux disease.

Table 4. Effect of Proton Pump Inhibitor Treatment on Salivary Pepsin Concentration and positive rate of Gastroesophageal Reflux Disease Symptoms in Patients With Non-erosive Reflux Disease and Extra-esophageal Symptoms

Group	Median (Q25-Q75) (ng/mL)	Positive rate
NERD	298.0 (39.0-468.5)	71.9%
NERD + PPI	7.0 (5.0-218.0)[a]	29.3%[a]
EES	230.0 (18.0-404.0)	68.2%
EES + PPI	16.0 (7.0-126.0)[b]	30.0%[c]

[a]Compared with NERD group, $P < 0.001$.
[b]Compared with EES group, $P = 0.002$.
[c]Compared with EES group, $P = 0.001$.
Q25, lower quartile; Q75, upper quartile; NERD, non-erosive reflux disease; NERD + PPI, NERD after proton pump inhibitor treatment; EES, extra-esophageal symptoms; EES + PPI, EES after PPI treatment.

The Meaningful Concentration of Pepsin for the Diagnosis of Erosive Esophagitis or Barrett's Esophagus

The meaningful SPCs for EE and BE as determined by ROC curve analysis. From the ROC curve, the meaningful concentration, which was the closest points to the upper left corner with high sensitivity and specificity, were 76 ng/mL for the diagnosis of EE, and 73 ng/mL for the diagnosis of BE.

Comparison of Pepsin in Saliva and Reflux Parameters in Patients With Non-erosive Reflux Disease

Reflux parameters used in 24-hour pH monitoring of the 64 NERD patients included the percentage of total time with pH < 4.0, percentage of total time with pH < 4.0 in the standing position, percentage of total time with pH < 4.0 in the decubitus posi-

Table 5. Positive Rates of Pathological Reflux Determined by 24-Hour Esophageal pH Monitoring in Patients With Non-erosive Reflux Disease

Indicator	Median (Q25-Q75)	Positive rate
Total time with pH < 4% (%)	1.5 (0.1-4.9)	40.6% (26/64)
Time with pH < 4% in standing position (%)	3.2 (0.1-6.9)	40.6% (26/64)
Time with pH < 4% in decubitus position (%)	0.0 (0.0-0.6)	20.3% (13/64)
Episodes of reflux with pH < 4.0 (times)	53.0 (20.0-70.3)	65.6% (42/64)
Episodes of reflux lasting for more than 5 minutes (times)	0.0 (0.0-4.0)	23.4% (15/64)
Longest duration of reflux (min)	4.0 (3.0-8.0)	12.5% (8/64)
Demeester score	13.6 (11.0-47.2)	43.8% (28/64)

tion, episodes of reflux with pH < 4.0, episodes of reflux lasting for ≥ 5 minutes, the longest duration of reflux, and the total score of Demeester ≥ 4.2% for percentage of total time with pH < 4.0, ≥ 6.3% for percentage of total time with pH < 4.0 in the standing position, ≥ 1.2% for percentage of total time with pH < 4.0 in the decubitus position, ≥ 50 for episodes of reflux with pH < 4.0, > 3.0 for episodes of reflux lasting for ≥ 5 minutes, ≥ 9.2 minutes for the longest duration of reflux, and ≥ 14.7 for total score of Demeester. The median (interquartile range) and the positive rates of of pathological reflux in patients with NERD are shown in Table 5. In this study, the positive rate of total DeMeester score ≥ 14.7 was 28 (43.8%) of 64 patients with NERD, however the positive rate of salivary pepsin in NERD patients was 71.9% (46/64), which was significantly higher than that of 24-hour pH monitoring ($\chi^2 = 9.06$, $P = 0.002$).

Discussion

This study showed that salivary pepsin has an important significance for the diagnosis of GERD and GERD-related disorders. It also has certain values for the differentiation between typical symptoms and esophageal hypersensitivity, and between EES and respiratory diseases. Its clinical values lie in providing guidance for diagnosis and treatment of GERD, reducing delayed management due to misdiagnosis, and proposing targeted treatments to improve the outcomes and reduce the incidence of drug resistance.

SPC and positive rates in EE, NERD, BE, EES, and EES + T-GERD groups were significantly higher than those in the HC group, suggesting that the salivary pepsin level is an intrinsic biomarker for the diagnosis of GERD and GERD-related disorders. A previous study reported a positive rate of 78.6% using salivary pepsin for the diagnosis of reflux-related diseases,[13] the positive rates of salivary pepsin in this study were 65.0-76.3% in NERD, EE, EES, and BE.

It has been reported that 30.0% of patients with refractory reflux symptoms has a FH, which is mainly caused by high esophageal hypersensitivity,[23] which is consistent with the low positive rate of salivary pepsin observed in this study. Moreover, a study found more episodes of reflux in patients with EES than those without reflux symptoms by 24-hour pH monitoring, among which episodes of reflux could be detected in 49.2% of patients with respiratory symptoms.[24] Therefore, for patients with long-term chronic dry cough, pharyngitis, asthma, hoarseness, and other respiratory manifestations and unresponsive to routine treatments, the possibility of EES should be considered.[25] Salivary pepsin level can quickly distinguish true reflux with non-reflux diseases, and is important for the diagnosis of GERD patients with predominant respiratory symptoms.[14-17,26] In this study, the positive rate of detecting EES was 68.2%, which was consistent with the observation of a previous study,[24] suggesting that salivary pepsin is of certain diagnostic value for the diagnosis of EES. In addition, the salivary pepsin level and positive rate in the BE group were significantly higher than those in the HC group in the present study, suggesting that pepsin reflux does play a significant role in the process of BE formation. Indeed, BE is closely related to acid reflux, pepsin reflux and/or bile reflux, all of which contribute to the esophageal injury and mucosal metaplasia.[27,28]

The interaction between GERD symptoms and mental stress has a synergistic impact on the life quality of patients, which was more closely related to the psychological factors than the severity of GERD symptoms.[29-31] For patients with refractory GERD with psychological disorders, psychological counseling, regulation of autonomic nerve function and anti-anxiety and anti-depression treatment should be added to acid suppression treatment, which can significantly improve the symptoms.[8,31-33] It has been demonstrated that the higher the level of anxiety and depression, the poorer, the effectiveness of acid-suppressing therapy, and thus psychological factors can be used as a predictor of anti-reflux treatment.[34-36] Therefore, patients with GERD with anxiety and depression were included in the present study. GERD-AD group was recruited

according to the GerdQ, endoscopy, and Hamilton Anxiety and Depression Rating Scale, and the GERD-AD group did not overlap with other groups. One of the aims of the present study was to explore the role of salivary pepsin in the pathogenesis of GERD with anxiety and depression, better understand the discrimination between GERD-AD and GERD without anxiety and depression and thus provide new therapeutic approaches for GERD with anxiety and depression. The present study showed that salivary pepsin levels and positive rates in the FH and GERD-AD group were significantly lower than those in patients with typical GERD symptoms without anxiety and depression, and about four-fifths of patients in GERD-AD group had negative salivary pepsin results, indicating the symptoms may be related to subjective discomfort caused by visceral hypersensitivity due to psychological disorders such as anxiety and depression with no true reflux. Previous studies have also shown that 30.0% of patients with refractory reflux symptoms are functional heartburns caused by esophageal hypersensitivity.[37,38]

This study showed that SPC and positive rates were significantly decreased after PPI treatment in the NERD and EES groups, suggesting that PPI treatment can reduce the SPC by inhibiting gastric acid secretion.[39,40] A multi-center study in Korea showed that the incidence of unresponsiveness after PPI treatment in patient with NERD was as high as 26.0%,[41] which was consistent with the positive rate of 29.3% in NERD with PPI intervention in this study. Previous studies have shown that there is no significant difference in the total episodes of reflux determined by 24-hour pH monitoring in unresponsive patients after PPI treatment, and the total episodes of weak-acid and non-acid reflux in refractory GERD patients were significantly more than that in the control group.[42,43] In addition, only 5.0% of reflux symptoms were related with acid reflux, while 16.7% were related to non-acid reflux, suggesting most unresponsive GERD patients after PPI treatment are caused by non-acid reflux.[42,43] Unresponsiveness after PPI treatment in patients with GERD is also associated with poor compliance, psychiatric comorbidities, and functional gastrointestinal disorders.[44,45] After taking PPI, gastric acid secretion is reduced, and the pH in the stomach rises to 4.0 or higher, inhibiting the activation of pepsinogen.[41,46] The current basic principle of drug therapy against GERD is to reduce gastric acid secretion and indirectly reduce the production of pepsin, thereby reducing the amount of gastric acid and pepsin refluxed to the esophagus, throat and other parts, resulting in significantly reduced clinical symptoms and the mucosa damages to the reflux site in GERD patients. [6,14,15]

This study showed that for NERD patients, the positive rate of salivary pepsin test was significantly higher than the pathologic reflux as determined by 24-hour pH monitoring (71.9% vs 43.8%), suggesting that salivary pepsin test can complement or combine with the 24-hour pH monitoring and improve the efficiency in the diagnosis of GERD. A recent study showed that 24-hour pH monitoring was less sensitive in the diagnosis of NERD,[47] which was consistent with our study. Also, Dulery et al[48] recently reported that negative results were obtained with 24-hour pH monitoring, while strong positive results were obtained with salivary pepsin test in saliva, sputum, middle ear fluid, and alveolar lavage fluid in some NERD patients. Rasijeff et al[49] showed that the positive rate of 24-hour pH monitoring was 49.0%, but 81.0% for the salivary pepsin test, indicating that a higher proportion of patients with reflux disease was detected by the pepsin test than 24-hour pH monitoring, which may reflect poor sensitivity of the 24-hour pH monitoring. Martinez et al[50] proposed to classify NERD into 3 sub-types: (1) typical GERD patients, in which 24-hour pH monitoring suggests that the symptoms are associated with pathological acid reflux, accounting for about 50.0% of NERD patients; (2) reflux-related patients, in which 24-hour pH monitoring does not show significant acid reflux, but the symptoms are related to acid reflux, accounting for about 18.0% of NERD patients; and (3) reflux-unrelated patients, in which symptoms are not related to acid reflux, but related to other forms of reflux or psychological factors, accounting for about 32.0% of NERD patients. These findings partially explain why there was lower positive rate for the diagnosis of NERD using 24-hour pH monitoring.

Pepsin is one of the main components in the reflux fluid, and also one of the main injury factors in GERD.[51] Previous studies have shown that esophageal and throat tissues were sensitive to pepsin, and a small amount of activated pepsin could cause inflammation in the mucosa of the esophagus and throat, thus causing subjective discomfort and symptoms.[52-54] A previous study by Li et al[55] showed that the intercellular spaces measurements were greater in EE and NERD groups than in FH and HC groups. Patients with NERD had a higher level of salivary pepsin compared to those with FH. SPCs correlated with severity of mucosal integrity impairment in the NERD group. Thus, low levels of salivary pepsin can help identify patients with FH; a higher pepsin concentration is more likely to be associated with an increased the severity of dilated intercellular spaces. Another study by Bardhan et al[56] also showed that higher concentrations of pepsin and frequency of exposure through an increased number of reflux events were more likely to result in injury of the susceptible laryngopharynx. Furthermore, the severity of reflux measured by the Reflux Findings Score (RFS)

was related to salivary pepsin, with higher RFS scores in subjects who had salivary pepsin compared to those who did not or who had a weak but significant correlation between the RFS and SPC. RFS with the presence of pepsin showed that patients with pepsin in the sputum had more significant symptoms and laryngoscopic signs of reflux than those who were pepsin negative in the sputum.[57]

The present study showed that pepsin detection in saliva has an important value for the diagnosis of GERD and GERD-related disorders, but there is no consensus concerning normal values, sensitivity and specificity to be used as a clinical tool for diagnosis of GERD. This non-invasive test still needs further verification to improve diagnosis for the pediatric population and patients following upper abdominal surgery, and further research is needed regarding the follow-up data to assess treatment outcomes after diagnostic decision based on salivary pepsin test.

In conclusion, salivary pepsin has an important significance for the diagnosis of GERD and GERD-related disorders. Salivary pepsin test has certain advantages over current diagnostic methods for GERD. It is expected that salivary pepsin test may become an important diagnostic tool for suspected GERD patients. Salivary pepsin and 24-hour pH monitoring may complement with each other to improve the diagnostic efficiency.

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