ORIGINAL ARTICLE

Pepsin in saliva for the diagnosis of gastro-oesophageal reflux disease

Jamal O Hayat,[1,2] Shirley Gabieta-Somnez,[1] Etsuro Yazaki,[1] Jin-Yong Kang,[2] Andrew Woodcock,[3] Peter Dettmar,[3] Jerry Mabary,[4] Charles H Knowles,[1] Daniel Sifrim[1]

[1]Centre for Digestive Diseases, Barts and the London School of Medicine and Dentistry, London, UK
[2]Department of Gastroenterology, St.George's, University of London, UK
[3]RD Biomed Limited, Hull, UK
[4]Sandhill Sci., Denver, Colorado, USA

Correspondence to
Dr Daniel Sifrim, Wingate Institute of Neurogastroenterology, 26 Ashfield Street, London E12AJ, UK;
d.sifrim@qmul.ac.uk

Received 19 February 2014
Revised 13 April 2014
Accepted 15 April 2014
Published Online First 8 May 2014

ABSTRACT

Objective Current diagnostic methods for gastro-oesophageal reflux disease (GORD) have moderate sensitivity/specificity and can be invasive and expensive. Pepsin detection in saliva has been proposed as an 'office-based' method for GORD diagnosis. The aims of this study were to establish normal values of salivary pepsin in healthy asymptomatic subjects and to determine its value to discriminate patients with reflux-related symptoms (GORD, hypersensitive oesophagus (HO)) from functional heartburn (FH).

Design 100 asymptomatic controls and 111 patients with heartburn underwent MII-pH monitoring and simultaneous salivary pepsin determination on waking, after lunch and dinner. Cut-off value for pepsin positivity was 16 ng/mL. Patients were divided into GORD (increased acid exposure time (AET), n=58); HO (normal AET and + Symptom Association Probability (SAP), n=26) and FH (normal AET and—SAP, n=27).

Results 1/3 of asymptomatic subjects had pepsin in saliva at low concentration (0(0–59)ng/mL). Patients with GORD and HO had higher prevalence and pepsin concentration than controls (HO, 237(52–311)ng/mL and GORD, 121(29–252)ng/mL)($p<0.05$). Patients with FH had low prevalence and concentration of pepsin in saliva (0(0–40) ng/mL). A positive test had 78.6% sensitivity and 64.9% specificity for diagnosis of GORD +HO (likelihood ratio: 2.23). However, one positive sample with >210 ng/mL pepsin suggested presence of GORD+HO with 98.2% specificity (likelihood ratio: 25.1). Only 18/84 (21.4%) of GORD+HO patients had 3 negative samples.

Conclusion In patients with symptoms suggestive of GORD, salivary pepsin testing may complement questionnaires to assist office-based diagnosis. This may lessen the use of unnecessary antireflux therapy and the need for further invasive and expensive diagnostic methods.

Significance of this study

What is already known on this subject?

- Diagnostic methods for gastro-oesophageal reflux disease (GORD) (empirical proton pump inhibitor treatment, GORD-specific questionnaires, endoscopy and ambulatory reflux monitoring), do not achieve high sensitivity and specificity.
- Pepsin detection in saliva has been proposed as a method for office-based GORD diagnosis, but there is no consensus concerning normal values, sensitivity and specificity of the test to be used as a clinical diagnostic tool for GORD.

What are the new findings?

- Pepsin can be found in saliva in healthy subjects and patients with heartburn, particularly during postprandial periods. Up to 1/3 of healthy asymptomatic subjects may have pepsin in saliva, but nearly all at concentrations below 200 ng/mL.
- Patients with reflux-related symptoms (GORD and hypersensitive oesophagus, HO) have a higher prevalence of pepsin in saliva and higher pepsin concentration than controls. Patients with functional heartburn have low prevalence and low concentration of pepsin in saliva.
- A positive saliva sample for pepsin (>16 ng/mL) has a sensitivity of 78.6% and a specificity of 64.9% for diagnosis of reflux-related symptoms. However, one sample positive with high pepsin concentration (>210 ng/mL) suggests that the symptoms are likely to be due to reflux with 98.2% specificity.
- Only 20% of patients with reflux-related symptoms (GORD+HO) had three negative samples.

How might it impact on clinical practice in the foreseeable future?

- We speculate that after appropriate validation, this non-invasive test can be used to improve diagnosis of GORD in the paediatric population, patients refractory to medical or surgical treatment, and in patients with extra-oesophageal symptoms attributed to GORD.

INTRODUCTION

The diagnosis and management of gastro-oesophageal reflux disease (GORD) seems simple, particularly in patients with typical symptoms and good response to treatment with proton pump inhibitors (PPI). Diagnostic methods to confirm or reject GORD include empirical PPI treatment, GORD specific questionnaires, endoscopy and ambulatory reflux monitoring. Unfortunately, these approaches do not achieve high sensitivity and specificity, particularly in patients with non-erosive reflux disease (NERD), and some of them are invasive and expensive.

Linked

▶ http://dx.doi.org/10.1136/gutjnl-2014-307485

CrossMark

To cite: Hayat JO, Gabieta-Somnez S, Yazaki E, *et al*. *Gut* 2015;**64**:373–380.

A systematic review suggested that the 'PPI test' lacked sensitivity and specificity as a clinical tool for diagnosis of GORD.[1] More recently, data from the Diamond Study showed that in patients with typical reflux symptoms, the sensitivity of the PPI test was 71% and the specificity was 44%[2] suggesting that the PPI test is unreliable for accurate diagnosis of GORD. Specific questionnaires have been developed for the diagnosis of GORD.[3] Structured questionnaires have a sensitivity and specificity of 63% and 67%, respectively (using endoscopy and pHmetry as the best available gold standard).[4]

Endoscopy is initially performed in patients with alarm signs or in patients with persistent symptoms despite PPI therapy. Endoscopy can provide a positive diagnosis of GORD in 30% of patients (when there is oesophagitis and/or Barrett's mucosa). However, most patients with symptoms suggesting GORD have no endoscopic evidence of mucosal damage and are categorised as having either NERD or functional heartburn (FH). In NERD patients, but not in FH, mucosal biopsies can show reflux-related microscopic changes.[5]

Reflux monitoring has good sensitivity and specificity in patients with oesophagitis but less sensitivity and reproducibility in patients with NERD.[6 7] In the Diamond Study, pHmetry failed to diagnose approximately one-third of patients with established reflux disease. Furthermore, studies have shown that there is day-to-day variability in the performance of pH studies, with the same patient demonstrating a normal study on one day and an abnormal study on another. The use of wireless Bravo pH monitoring and impedance-pHmetry increases the sensitivity and leads to more accurate phenotyping,[8–10] however, these are invasive and expensive techniques that require endoscopy or nasogastric intubation which is uncomfortable and inconvenient for a proportion of patients.

In routine clinical practice, there is a need to distinguish patients with symptoms that are 'due' to reflux (either increased reflux or hypersensitivity to a normal amount of reflux) from patients with symptoms suggestive of reflux but 'not due' to reflux (ie, FH). The later should not receive prolonged PPI treatment, nor be offered antireflux surgery. Therefore, better tools for GORD diagnosis are warranted, particularly, if they have low cost, are non-invasive and can be performed in a primary care, ideally office, setting.

Pepsin is a proteolytic enzyme whose precursor pepsinogen is released solely by gastric chief cells. Its presence in the oesophagus or more proximally (pharynx or airways) suggests gastro-oesophageal reflux (GOR). To date, pepsin has been detected in saliva, and secretion samples from trachea, lung, sinus, middle ear and exhaled breath condensate.[11–15] Pepsin detection in saliva has previously been proposed as a method for GORD diagnosis. However, investigators used different protocols for saliva sampling (timing and number), methodologies for pepsin detection and cut-off values.[12 16–24] To date, there is no consensus concerning normal values for salivary pepsin determination in healthy asymptomatic subjects. As a consequence, the use of pepsin determination in saliva as a clinical diagnostic tool for GORD, requires further validation, particularly if the test is proposed as an 'office based method' to distinguish patients with symptoms 'due' to reflux from patients with similar symptoms but not reflux-related.

The aims of this study were (1) to assess the relationship between pepsin in saliva and gastro-oesophageal reflux, by performing multiple determinations of pepsin in saliva during ambulatory reflux monitoring, (2) to establish normal values of salivary pepsin in a large cohort of healthy asymptomatic subjects and (3) to determine the sensitivity and specificity of salivary pepsin determination for positive diagnosis of patients whose symptoms are related to reflux, that is, those with GORD or hypersensitive oesophagus (HO), and exclusion of patients with FH.

METHODS

Subjects

Studies were performed in 104 asymptomatic healthy volunteers (mean age 30.7 years (range 19–55), 55F: 45M) recruited by advertisements placed at St George's University of London, and 134 consecutive patients with typical GORD symptoms (predominant heartburn with or without regurgitation) (mean age 49.7 years (range 23–77), 62F: 49M) referred to the Upper Gastrointestinal Physiology Unit at the Royal London Hospital (Barts Health NHS Trust) for reflux assessment. Patients were included if their primary complaint was heartburn. We excluded patients with a history of previous oesophageal/gastric surgery, or a known oesophageal motor disorder (eg, achalasia, scleroderma). The study was approved by the London City and East Research Ethics Committee (ref: 10/H0703/14).

Questionnaire

All subjects were asked to complete the Reflux Disease Questionnaire (RDQ).[25] Questions are divided into three domains—heartburn, regurgitation and dyspepsia. The 'GORD-RDQ' score is determined by the sum of the items within the heartburn and regurgitation dimensions and gives a score between 0 and 40.

Reflux monitoring (impedance-pHmetry)

Reflux monitoring was performed using impedance-pHmetry (MII-pH) (Sandhill Scientific, Highlands Ranch, Colorado, USA). The MII-pH catheter incorporates 2 pH sensors and six impedance channels. The catheter was positioned such that the proximal pH sensor was located in the oesophagus 5 cm above the proximal border of the lower oesophageal sphincter (LOS) (determined manometrically), and the distal (gastric) pH sensor was placed 10 cm below the LOS. The six impedance sensors were located in the oesophageal body at 3, 5, 7, 9, 15 and 17 cm above the LOS. All patients were studied 'off PPI' (for at least 7 days). All were asked to record their typical symptoms, mealtimes, as well as periods of recumbent and upright positions. they were asked to have lunch starting between 12:00 and 13:30, and dinner between 18:00 and 19:30. Each tracing was manually edited by either JOH or DS to ensure accurate reflux detection. Reflux episodes were characterised as either pure liquid or mixed liquid-gas, and as either acid or weakly acid according to published consensus criteria.[26] Proximal reflux was defined as episodes reaching the 15 cm impedance channel. Symptom Association Probability (SAP) was used to characterise the association between reflux and symptoms.[27]

Salivary pepsin

Collection

Subjects collected saliva on waking, 1 h after finishing lunch and 1 h after finishing dinner during the 24 h ambulatory MII-pH monitoring period. Subjects were asked to take the early morning sample before eating or drinking and before brushing their teeth. Saliva was collected into tubes containing 0.5 mL of 0.01 M citric acid. Subjects returned the samples together with the reflux monitoring system. Samples were refrigerated at 4°C and analysed within 2 days of collection.

Analysis

Analysis was performed blinded to whether subjects were healthy controls or GORD patients and to any reflux monitoring parameter or RDQ scores. Collection tubes were centrifuged at 4000 rpm for 5 min until a clear supernatant layer was seen. If not, samples were centrifuged again, and 80 µL from the surface layer of the centrifuged sample was drawn up into an automated pipette. The 80 µL sample was transferred to a screw-top microtube containing 240 µL of migration buffer solution. This sample was mixed with a vortex mixer for 10 s. A second pipette was used to transfer 80 µL of the sample to the circular well of a Lateral Flow Device (LFD) containing two unique human monoclonal antibodies; one to detect and one to capture pepsin in the saliva sample (Peptest, RDBiomed). The lower limit for accurate detection of pepsin (as determined by the manufacturer) was set at 16 ng/mL. We used this value as a cut-off to consider a saliva sample positive for pepsin. Therefore, all samples with determinations below this threshold were considered to have 0 ng/mL in the results.

Statistical analysis

Data were expressed as mean±SEM for variables with normal distribution, and median (IQR) for variables with non-normal distribution. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's Test for normal distributed data and the Kruskall–Wallis Test with Dunns comparison for non-normal data. Correlations between pepsin concentration and reflux variables were assessed with Pearson's or Wilcoxon rank sum tests when appropriate. A p value of <0.05 was considered significant. Receiver Operator Characteristic curves were constructed to determine and compare the sensitivity and specificity of different pepsin cut-off concentrations and their predictive value to diagnose or refute the diagnosis of GORD and reflux-related symptoms. Likelihood ratios were calculated to aid interpretation using the following definitions: >10: large and often conclusive increase in the likelihood of disease; 5–10: moderate increase in the likelihood of disease; 2–5: small increase in the likelihood of disease; 1–2: minimal increase in the likelihood of disease; 1: no change in the likelihood of disease; 0.5–1.0: minimal decrease in the likelihood of disease; 0.2–0.5: small decrease in the likelihood of disease; 0.1–0.2: moderate decrease in the likelihood of disease; <0.1: large and often conclusive decrease in the likelihood of disease. Data were analysed using Stata V.10, and Prism V.5.0, GraphPad.

RESULTS

Patients

In total, 238 people were recruited (104 healthy volunteers and 134 patients with heartburn) (figure 1). Four of the healthy subjects were not able to tolerate the full 24 h MII-pH recording period, and 13 asymptomatic subjects had increased gastro-oesophageal reflux[28] and were excluded from the analysis. Therefore, 87 asymptomatic healthy subjects served as controls. There were insufficient data for 23 of the patients (reasons were: not collecting all three saliva samples, unable to tolerate full 24 h MII-pH recording period, and technical failure of the MII-pH probe to record data). In total, 111 symptomatic patients were included in the analysis.

Patients were classified according to the oesophageal acid exposure time (AET) and reflux-symptom association analysis. All patients with total AET >4.2% were classified as having GORD (n=58). Patients with a normal AET but with a positive symptom association analysis (SAP >95%) were classified as having HO (n=26). Patients with a normal AET and a negative symptom association analysis (SAP <95%) were classified as having FH (n=27).[29]

The mean age of the controls (30.7 years (range 19–55), was significantly lower than GORD patients 53.2 years (23–77, 30F: 28M), HO patients 42.8 years (25–69, 18F: 8M), and FH patients 49.5 years (25–71, 14F: 13M). There was no significant gender difference between groups.

Prevalence of positive pepsin detection in saliva

Totally, 33/87 healthy asymptomatic subjects had one or more saliva samples positive for pepsin (21% of all samples were positive for pepsin) (table 1). Compared to healthy controls, patients with heartburn had a significant higher prevalence of saliva

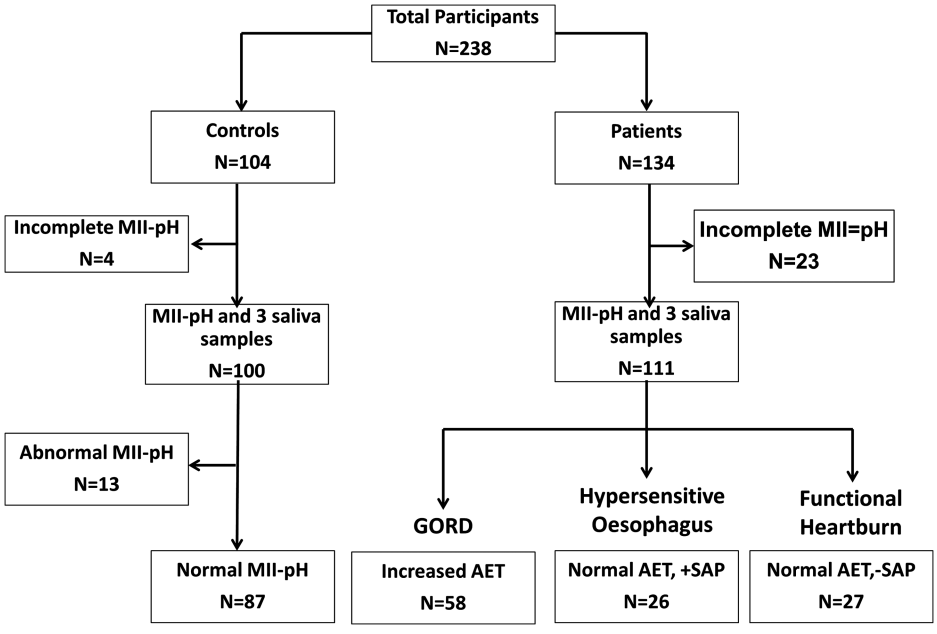

Figure 1 Flowchart showing participant recruitment and patient classification.

samples positive for pepsin. 75/111 patients had one or more samples positive (40.1% of all samples were positive for pepsin), p<0.0001.

Among patients with heartburn, those with GORD had a high prevalence of pepsin detection, with 45/58 subjects having one or more samples positive (45% of all samples). Similarly, HO patients had high prevalence of positive pepsin in saliva (21/26 patients, 51% of all samples positive). By contrast, patients with FH had a significantly lower prevalence of pepsin detection (9/27 patients with pepsin detected in one or more samples, 21% of all samples positive) than either patients with GORD (p<0.0002), or patients with HO (p<0.0008). The prevalence of pepsin detection was similar between FH patients and asymptomatic controls (table 1). Very few subjects had all three samples positive for pepsin (5/87 in controls, 5/58 in GORD, 6/26 in HO and 0/27 in FH).

Pepsin concentration in saliva

The highest pepsin concentration in saliva (out of the three samples) was determined for each subject. Considering all subjects in each group, pepsin concentration was significantly higher in patients with heartburn 75 (0–248) ng/mL compared to controls 0 (0–59) ng/mL, p<0.05. The pepsin concentration in GORD patients was 121 (29–252) ng/mL and 237 (52–311) ng/mL in HO. By contrast, in patients with FH, saliva pepsin concentration was significantly lower (0 (0–40) ng/mL) (p<0.05) and similar to healthy controls (figure 2 and table 1). There were five patients with HO who had increased non-acid reflux, with a median pepsin concentration of 188 (0–256.5).

Considering only subjects with positive samples (ie, >16 ng/mL), pepsin concentration in GORD patients was 126 (49.75–246.3) ng/mL, and it was significantly higher in patients with HO 237 (81.25–305) ng/mL (p<0.05). By contrast, positive samples in patients with FH and asymptomatic controls had significantly lower pepsin concentration (40 (30–68) ng/mL) and 56 (27.5–120.5) ng/mL, respectively. Salivary pepsin concentration was not significantly different between younger (<55 years) and older patients (>55 years) (124 (43–240) ng/mL vs 142 (70–270) ng/mL, respectively. Younger patients had significantly higher salivary pepsin concentration than controls, p<0.0004.

Timing of positive pepsin samples

The prevalence of positive pepsin samples and concentration of pepsin in saliva were significantly lower in the morning waking samples compared to postprandial samples, in controls and patient groups. Interestingly, postprandial pepsin measurements discriminated better than morning samples between GORD +HO patients on the one hand, and FH patients/healthy controls on the other hand. Pepsin was significantly more likely to be detected in the postprandial period in patients with GORD (57.8% postprandial samples vs 21% morning samples, p<0.03) and HO patients (69.2% postprandial vs 23% morning p<0.0002). By contrast, in FH patients, pepsin was positive in 20.3% of postprandial samples and 18% of morning samples. There was a significant intraindividual variability in salivary pepsin concentration variability over the 24 h (figure 3).

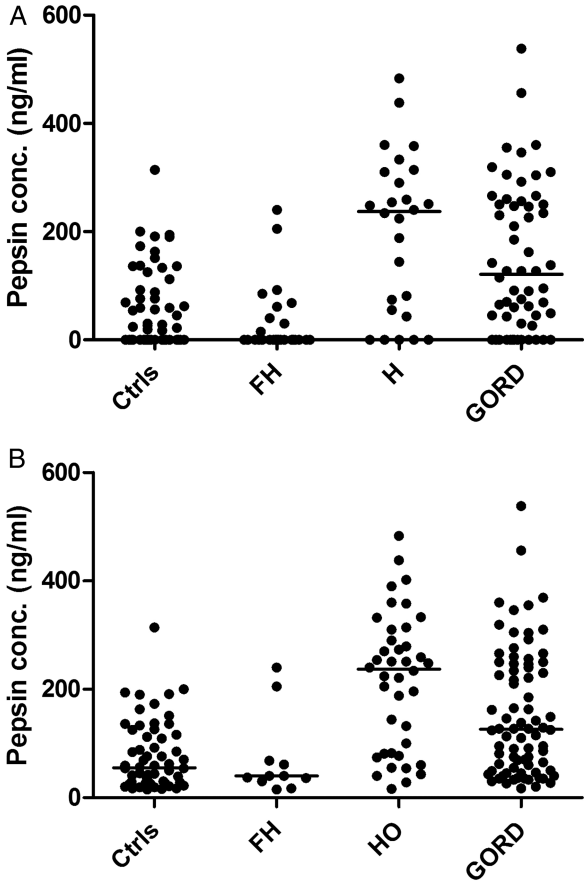

Figure 2 (A) Highest pepsin concentration in all subjects. (B) Highest pepsin concentration of positive samples in controls and different patient groups. Horizontal bars represent median values for each group.

Correlation between pepsin in saliva and reflux parameters

Concentration of pepsin in saliva had a significant but weak positive correlation with 24 h AET (r=0.316, p<0.0001), and total number of reflux episodes (r=0.249, p<0.0004).

Table 1 Values for the prevalence and concentration of salivary pepsin for controls and each group of patients

	Prevalence of positive samples (%)	Mean concentration of positive samples (±SEM)	Median concentration (25–75th centiles), 95th centile	Highest pepsin concentration (median (25–75th centiles), 95th centile
Controls n=87	21	76.6±8.3	55 (26.75–118.3), 194.3	0 (0–59), 190.6
Functional heartburn n=27	21	71.7±23.1	40 (30–68), 240	0 (0–40), 226
Hypersensitive oesophagus n=26	51	214.7±19.8	237 (81.25–305), 436.2	237 (0–311), 467.3
Gastro-oesophageal reflux disease n=58	45	153.7±13.1	126 (49.7–246.3), 360.5	121 (29–251.5) 364.8

Concentrations are given in ng/mL.

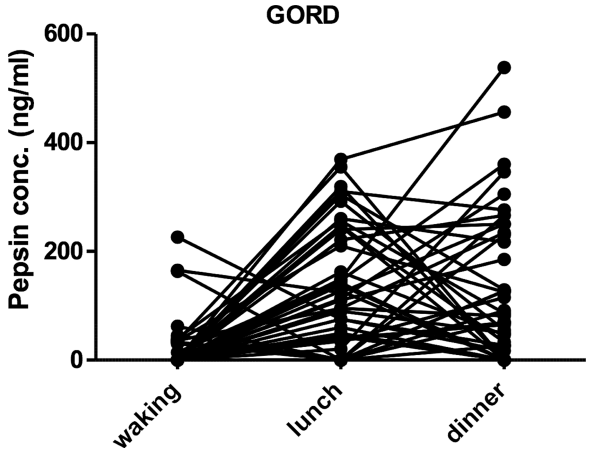

Figure 3 Intraindividual variability in salivary pepsin concentration during a 24 h period in patients with gastro-oesophageal reflux disease.

The number of postprandial reflux episodes was significantly higher in GORD patients, 14 (7–17) than in HO, 9 (7–16.5), FH patients 9 (4–13) or controls, 7 (4–10), ($p<0.021$). Patients with more than 15 postprandial reflux episodes (95th percentile of healthy controls) had a significantly higher pepsin concentration, 112 (56–290) ng/mL than subjects with less than 15 reflux episodes 0 (0–127) ng/mL $p<0.0001$.There was a significant but weak correlation between number of postprandial reflux events and concentration of pepsin in saliva ($r=0.3907$, $p<0.0001$). There was no correlation between number of proximal reflux episodes and pepsin concentration in saliva.

Pepsin concentration in saliva to differentiate patients with GORD, or patients with reflux-related symptom (GORD+HO) from patients with FH

Receiver operating characteristics analysis was used to identify the best cut-off value of saliva pepsin concentration, to differentiate GORD patients from FH patients and controls, (figure 4A). The area under the receiver operating characteristic curve had a value of 0.7725 ± 0.04 (95% CI 0.6937 to 0.8512, $p<0.0001$). A saliva pepsin concentration of >210 ng/mL had a sensitivity of 37.9%, and a specificity of 98.2%. We also identified the best cut-off value of saliva pepsin concentration to differentiate all patients with reflux-related symptoms (GORD+HO) patients from FH+controls patients, (figure 4B). The area under the receiver operating characteristic curve had a value of 0.8034 ± 0.04 (95% CI 0.719 to 0.8873, $p<0.0001$). A saliva pepsin concentration of >210 ng/mL had a sensitivity of 44%, and a specificity of 98.2%.

Table 2 shows a range of saliva pepsin concentrations and their usefulness in identifying patients with heartburn 'due' to reflux (GORD or HO). The gold standard, that is, 'true' reflux-related symptoms, was based on positive MII-pH monitoring (increased AET and/or positive SAP).

Three determinations of pepsin in saliva during 24 h, allowed for the following scenarios. The majority of controls and patients with FH (74/114, (65%) had three negative samples. By contrast, only 18/84 (21.4%) of GORD+HO patients had three negative samples. If at least one sample was positive (>16 ng/mL), the test showed a sensitivity of 78.6% and specificity of 64.9%, with a negative predictive value (NPV) of 80.4%. When one sample was positive, the usefulness of the test depended on the pepsin concentration. At a low concentration of <100 ng/mL, the sensitivity and specificity were 70.2% and 73.6%, respectively, with low positive likelihood ratio. With these inconclusive results, further diagnostic investigations may be warranted. With a moderate concentration of between 100 and 200 ng/mL, there was a 80% positive predictive value (PPV), and while the diagnosis was likely to be reflux, further conclusive evidence may be needed in patients not responding to treatment (likelihood ratio >5 ie, moderate increase in the likelihood of disease). With a higher concentration (>210 ng/mL) the specificity and PPV were above 94.5%, and the likelihood ratio was >10 (25.1) suggesting a large and often conclusive increase in the likelihood of disease.

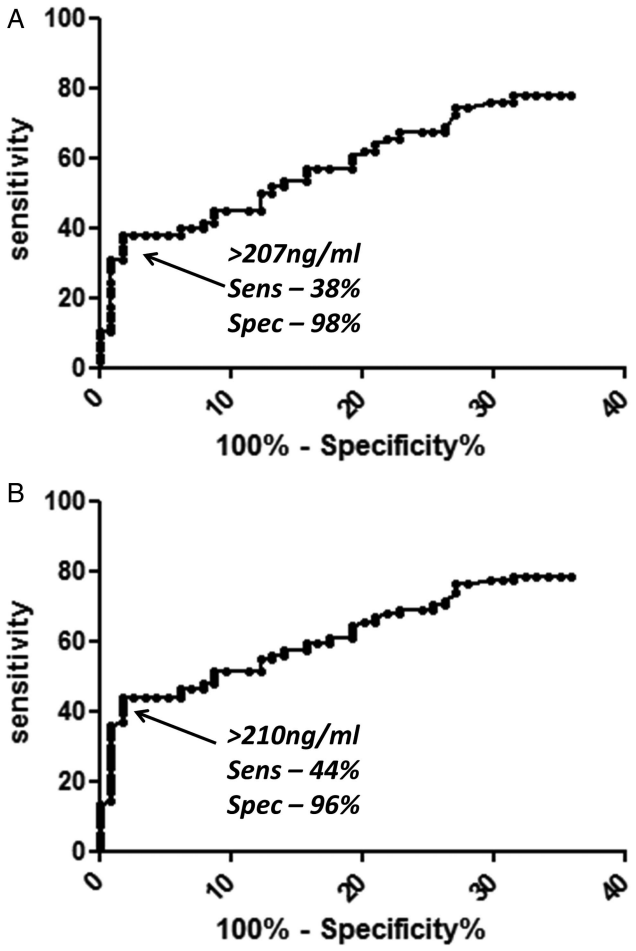

Figure 4 Receiver operating characteristics curve of the highest pepsin concentration between patients with (A) gastro-oesophageal reflux disease (GORD) only and (B) GORD+HO versus controls+FH patients. FH, functional heartburn; HO, hypersensitive oesophagus.

DISCUSSION

To confirm or reject the diagnosis of GORD is clinically very relevant in order to avoid unnecessary, prolonged and expensive treatments with PPIs or antireflux surgery. This could be the case in the initial assessment of patients with heartburn, in the assessment of patients with atypical symptoms attributed to reflux, in patients whose symptoms are refractory to PPI and, lastly, in patients with persistent reflux symptoms after antireflux surgery. Unfortunately, there is no perfect technique for diagnosis of GORD, as most methods have moderate sensitivity and specificity, or are invasive and expensive.

Recently, pepsin determination in saliva has been proposed as a non-invasive diagnostic method for reflux disease.[24] In this

Table 2 Proportions of patients with positive samples, sensitivities, specificities, positive and negative predictive values and likelihood ratios for a range of pepsin concentrations and their ability to identify patients with GORD+HO

	GORD+HO (%)	FH+controls (%)	Sensitivity %	Specificity %	PPV %	NPV %	+ve likelihood ratio	-ve likelihood ratio
At least 1 sample +ve > 16 ng/mL	66/84 (78.6)	40/114 (35.0)	78.5	64.9	62.2	80.4	2.23	0.33
At least 1 sample +ve > 50 ng/mL	59/84 (70.2)	30/114 (26.3)	70.2	73.6	66.3	77.1	2.67	0.40
At least 1 sample +ve > 100 ng/mL	47/84 (56.0)	16/114 (14.0)	55.9	85.9	74.6	72.5	3.98	0.51
At least 1 sample +ve > 150 ng/mL	40/84 (47.6)	10/114 (8.7)	47.6	91.2	80.0	70.2	5.42	0.57
At least 1 sample +ve > 210 ng/mL	37/84 (44.0)	2/114 (1.7)	44.0	98.2	94.8	70.4	25.1	0.56

FH, functional heartburn, GORD, gastro-oesophageal reflux disease; HO, hypersensitive oesophagus; NPV, negative predictive value; PPV, positive predictive value.

study, we determined the sensitivity and specificity of salivary pepsin for the positive diagnosis or exclusion of reflux-related symptoms in patients with heartburn. Our results showed (1) that pepsin can be found in saliva in healthy subjects and patients with heartburn, particularly during postprandial periods, (2) up to 1/3 of healthy asymptomatic subjects may have pepsin in saliva but at concentrations below 200 ng/mL, (3) patients with reflux-related symptoms (GORD and HO) have a higher prevalence of pepsin in saliva and higher pepsin concentration than controls, (4) patients with heartburn not related to reflux (FH) have low prevalence and low concentration of pepsin in saliva, (5) one saliva sample positive for pepsin has a sensitivity of 78.6% and a specificity of 64.9% for diagnosis of reflux-related symptoms, (6) approximately 20% of GORD+HO patients had three negative samples and (7) one positive sample with high pepsin concentration (>210 ng/mL) results in a 98.2% specificity for GORD+HO with a very high PPV (94.8%) and likelihood ratio (25.1).

The sensitivity and specificity of salivary pepsin detection are far from perfect but similar to those achieved by other methods for GORD diagnosis, with the advantage that this technique is non-invasive and inexpensive.

A frequent question that clinicians are faced with is whether a patient's symptoms are 'due to reflux' (initial diagnosis or persistence in spite of treatment), or 'not due to reflux' (FH, dyspepsia or in case of atypical symptoms, due to any other reason, ie, smoking, infection, allergy, or medications). For patients with normal endoscopy, the question is: does the patient have NERD or FH? Given the relatively favourable side-effect profile of PPIs, standard clinical practice is to initiate a trial of acid suppressant. If the patient does not respond to treatment, the clinician usually requires reflux monitoring with impedance-pHmetry, prolonged wireless pHmetry or endoscopy. Very recently, characteristic features on oesophageal endoscopic biopsies have been proposed for a differential diagnosis between NERD and FH.[30]

The current study showed that (similar to other methods) salivary pepsin test has moderate sensitivity and specificity for diagnosis of patients with GORD or patients with reflux related symptoms (similar to other diagnostic methods), however, having an office-based method to help identify or exclude these patients is very attractive, particularly if it is non-invasive, fast and inexpensive. Furthermore, it can help the clinician to offer the patient a potential prediction of response to PPIs. For example, in an individual with GORD symptoms but negative pepsin testing, the clinician may still consider an initial PPI treatment, but can already propose to the patient alternative medication such as baclofen (to reduce TLOSRs) or a neuromodulator (to reduce sensitivity) in case of PPI failure. Patients failing empirical PPI therapy were not included in the current study. We considered mandatory, as a first step, to assess the diagnostic efficacy of detecting salivary pepsin in a well-characterised group of patients with typical reflux symptoms and pH, confirmed GORD. An adjunctive study evaluating patients who fail PPI treatment will be required before the test can be recommended for general clinical use, and/or assessment of more difficult patients (ie, PPI refractory or extraoesophageal GORD).

So far, two studies have assessed pepsin in saliva in patients with heartburn. In the study by Saritas Yuksel *et al*,[24] the prevalence of positive salivary pepsin was low in all tested subjects: 12% in controls and up to 47% in GORD patients with oesophagitis plus pH abnormality. Saliva samples were only taken at one unspecified time point and concentrations were not determined. De Bartoli *et al*[31] sampled saliva at the time of symptoms in a small group of patients with typical GORD symptoms. Saliva was positive for pepsin in 94% of patients with GORD, 58% of HO patients, and always negative in patients with FH.

The morning saliva samples were less likely to be positive in all groups. By contrast, similar to reflux monitoring, patients had more often postprandial positive samples, and there was a significant intraindividual variability with subjects having high pepsin concentration postlunch, and negative samples postdinner, or vice versa. Due to this variability, we suggest the need for multiple postprandial sampling during the 24 h periods.

In our study, the prevalence of at least one positive pepsin sample was 78% in GORD, 80% in HO and 33% in FH, but we found that concentration of pepsin rather than simple positivity was of greater diagnostic use. We found that samples were mainly positive in the postprandial periods. This might be explained by increased postprandial gastric pepsin concentration and volume of reflux.[32] Based on our results, only postprandial saliva sampling would be recommended in the clinical office setting.

Pepsin was detected in the saliva of one-third of healthy asymptomatic individuals. This finding suggests that physiological reflux may bring small amounts of pepsin into the oral cavity. The difference with symptomatic patients is mainly quantitative (concentration). Patients with reflux-related symptoms (GORD and HO) had higher prevalence of positive samples and higher concentration than controls, regardless of the acidity of the refluxate, that is, what was also observed in patients with symptoms associated to non-acid reflux detected by impedance. This can be related to more reflux or more pepsin in the stomach. However, previous studies have shown similar gastric pepsin secretion rates in healthy controls and patients with oesophagitis.[33]

Similar to the previous studies, patients with FH had low pepsin detection rates and low concentration. This is clinically relevant, because having all negative pepsin samples suggests that symptoms might not due to reflux and therefore, avoids unnecessary prolonged PPI treatment and antireflux surgery.

There are several different isoforms of pepsin, most produced solely in the stomach, however, pepsin C may be produced by

pneumocytes.[34] Therefore non-specific pepsin assays may give false positive results. In our study, we used an indirect sandwich ELISA to detect pepsin. This is designed to overcome cross-reactivity with pepsinogen and other proteins with the use of two unique human monoclonal antibodies to pepsin 3b which is produced only in the stomach.[14]

We found a weak correlation between salivary pepsin and reflux parameters Oesophageal acid exposure and number of reflux episodes express the degree or 'burden' of exposure of the oesophageal mucosa to gastric contents. By contrast, an increased concentration of pepsin in an individual saliva sample is more a qualitative measurement and gives an indication that reflux has occurred. Additionally, other factors are likely to contribute to the concentration of pepsin in saliva. These include the intraindividual and interindividual variability in salivary flow rate and composition, as well as swallowing frequency.

In spite of having a weak correlation with reflux parameters, determination of pepsin in saliva can be a non-invasive inexpensive method that can help to confirm or exclude the diagnosis of GORD. In our study, all negative saliva samples suggested an 80% probability of diagnosing FH, whereas a positive sample with more than 210 ng/mL suggested reflux-related symptoms with a probability of 95%. Using these criteria, we could classify 75/111 patients. The remaining 36 patients would require further investigations.

In a recent review on the role of questionnaires for diagnosis of GORD in primary care,[35] Vakil N suggests that diagnosis of uncomplicated GORD, should use a strategy that keeps costs and inconvenience to the patient at a minimum.[35] We suggest that a combination of specific GORD questionnaires and determination of salivary pepsin can achieve such goals.

One limitation of our study was the 'gold standard' selected for consideration of 'real' GORD. It is known that catheter-based reflux monitoring can fail to diagnose patients with established reflux disease, and patients can show an important day-to-day variability. Another limitation was the failure to recruit healthy volunteers of an age range identical to the patient group. The younger age of the healthy subjects could be argued to have led to lower pepsin concentrations than in patients. However, we found no difference in acid exposure, number of reflux episodes and salivary pepsin concentration between patients in the age range of 19–55 years, and patients over 55 years. Furthermore, the younger GORD+HO patients had significantly higher salivary pepsin concentration when compared to selected age-matched controls. We acknowledge, however, that this still remains a potential confounder to results.

Our study did not include standardisation of meals but only meal times. Salivary sampling occurred during a patient's clinical reflux monitoring, which is generally performed without meal standardisation to simulate as much as possible the patient's real life. Another limitation of our study is the lack of follow-up data to assess treatment outcomes after diagnostic decision based on salivary pepsin testing. Furthermore, we assume that performance of the test in patients with heartburn can predict results in patients with atypical symptoms or patients with persistent symptoms in spite of PPI or antireflux surgery. Ongoing studies in these populations will unravel the real usefulness of salivary pepsin detection in these common clinical scenarios.

In summary, our findings showed a higher prevalence and concentration of salivary pepsin in patients with GORD or HO compared with FH patients and healthy controls. In patients with symptoms suggestive of GORD, salivary pepsin testing may complement questionnaires to assist office-based diagnosis. This may lessen the use of unnecessary anti-reflux therapy and the need for further invasive and expensive diagnostic methods.

Correction notice Table 2 has been updated along with related sentences throughout the article since published Online First.

Contributors JOH, EY, J-YK, SG-S: acquisition, analysis and interpretation of data; drafting of the manuscript. JM: review of impedance-pHmetry analysis. CHK: statistical analysis, critical revision of the manuscript. AW and PD: analysis of saliva samples for pepsin. DS: study design and concept, interpretation of data, overall study supervision final manuscript.

Funding Impedance-pH catheters were supplied by Sandhill Scientific, USA. Pepsin test kits were provided by RD Biomed Limited, and analysis determinations were performed by AW (RD Biomed Limited). Neither Sandhill Sci nor RD Biomed Limited had a role in the study design, conduct, statistical analysis, interpretation and manuscript preparation.

Competing interests Andrew Woodcock is employed by RD Biomed Limited. PD is director of RD Biomed (RDB). RDB own the patent (GB 1208663.3) and have developed and own all of the intellectual property surrounding the two monoclonal antibodies described in Peptest. JM was Vice President of Sandhill Scientific. DS receives a research grant from Sandhill Scientific.

Patient consent Obtained.

Ethics approval London, City and East Research Ethics Committee (ref: 10/H0703/14).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

REFERENCES

1 Numans ME, Lau J, de Wit NJ, *et al*. Short-term treatment with proton-pump inhibitors as a test for gastroesophageal reflux disease: a meta-analysis of diagnostic test characteristics. *Ann Intern Med* 2004;140:518–27.
2 Bytzer P, Jones R, Vakil N, *et al*. Limited ability of the proton-pump inhibitor test to identify patients with gastroesophageal reflux disease. *Clin Gastroenterol Hepatol* 2012;10:1360–6.
3 Vakil NB, Halling K, Becher A, *et al*. Systematic review of patient-reported outcome instruments for gastroesophageal reflux disease symptoms. *Eur J Gastroenterol Hepatol* 2013;25:2–14.
4 Dent J, Vakil N, Jones R, *et al*. Accuracy of the diagnosis of GORD by questionnaire, physicians and a trial of proton pump inhibitor treatment: the diamond study. *Gut* 2010;59:714–21.
5 Savarino E, Zentilin P, Mastracci L,, *et al* Microscopic esophagitis distinguishes patients with non-erosive reflux disease from those with functional heartburn. *J Gastroenterol* 2013;48:473–82.
6 Vaezi MF, Schroeder PL, Richter JE. Reproducibility of proximal probe pH parameters in 24-hour ambulatory esophageal pH monitoring. *Am J Gastroenterol* 1997;92:825–9.
7 Kahrilas PJ, Quigley EM. Clinical esophageal pH recording: a technical review for practice guideline development. *Gastroenterology* 1996;110:1982–96.
8 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.
9 Bredenoord AJ, Weusten BL, Timmer R, *et al*. Addition of esophageal impedance monitoring to pH monitoring increases the yield of symptom association analysis in patients off PPI therapy. *Am J Gastroenterol* 2006;101:453–9.
10 Zerbib F, Roman S, Ropert A, *et al*. Esophageal pH-impedance monitoring and symptom analysis in GERD: a study in patients off and on therapy. *Am J Gastroenterol* 2006;101:1956–63.
11 Farhath S, He Z, Nakhla T, *et al*. Pepsin, a marker of gastric contents, is increased in tracheal aspirates from preterm infants who develop bronchopulmonary dysplasia. *Pediatrics* 2008;121:e253–9.
12 Stovold R, Forrest IA, Corris PA, *et al*. Pepsin, a biomarker of gastric aspiration in lung allografts: a putative association with rejection. *Am J Respir Crit Care Med* 2007;175:1298–303.
13 Crapko M, Kerschner JE, Syring M, *et al*. Role of extra-esophageal reflux in chronic otitis media with effusion. *Laryngoscope* 2007;117:1419–23.
14 Knight J, Lively MO, Johnston N, *et al*. Sensitive pepsin immunoassay for detection of laryngopharyngeal reflux. *Laryngoscope* 2005;115:1473–8.
15 Yates DH, Krishnan A, Chow S, *et al*. Non-invasive assessment of exhaled biomarkers in lung transplantation. *J Breath Res* 2011;5:024001,7155/5/2/024001.
16 Kim TH, Lee KJ, Yeo M, *et al*. Pepsin detection in the sputum/saliva for the diagnosis of gastroesophageal reflux disease in patients with clinically suspected atypical gastroesophageal reflux disease symptoms. *Digestion* 2008;77:201–6.
17 Grabowski M, Kasran A, Seys S, *et al*. Pepsin and bile acids in induced sputum of chronic cough patients. *Respir Med* 2011;105:1257–61.

18 Potluri S, Friedenberg F, Parkman HP, *et al*. Comparison of a salivary/sputum pepsin assay with 24-hour esophageal pH monitoring for detection of gastric reflux into the proximal esophagus, oropharynx, and lung. *Dig Dis Sci* 2003;48:1813–17.
19 Wang L, Liu X, Liu YL, *et al*. Correlation of pepsin-measured laryngopharyngeal reflux disease with symptoms and signs. *Otolaryngol Head Neck Surg* 2010;143:765–71.
20 Ward C, Forrest IA, Brownlee IA, *et al*. Pepsin like activity in bronchoalveolar lavage fluid is suggestive of gastric aspiration in lung allografts. *Thorax* 2005;60:872–4.
21 McNally P, Ervine E, Shields MD, *et al*. High concentrations of pepsin in bronchoalveolar lavage fluid from children with cystic fibrosis are associated with high interleukin-8 concentrations. *Thorax* 2011;66:140–3.
22 Starosta V, Kitz R, Hartl D, *et al*. Bronchoalveolar pepsin, bile acids, oxidation, and inflammation in children with gastroesophageal reflux disease. *Chest* 2007;132:1557–64.
23 Krishnan U, Mitchell JD, Messina I, *et al*. Assay of tracheal pepsin as a marker of reflux aspiration. *J Pediatr Gastroenterol Nutr* 2002;35:303–8.
24 Saritas Yuksel E,, Hong SK, Strugala V, *et al*. Rapid salivary pepsin test: Blinded assessment of test performance in gastroesophageal reflux disease. *Laryngoscope* 2012;122:1312–16.
25 Shaw MJ, Talley NJ, Beebe TJ, *et al*. Initial validation of a diagnostic questionnaire for gastroesophageal reflux disease. *Am J Gastroenterol* 2001;96:52–7.
26 Sifrim D, Castell D, Dent J, *et al*. Gastro-oesophageal reflux monitoring: review and consensus report on detection and definitions of acid, non-acid, and gas reflux. *Gut* 2004;53:1024–31.
27 Weusten BL, Roelofs JM, Akkermans LM, *et al*. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. *Gastroenterology* 1994;107:1741–5.
28 Shay S, Tutuian R, Sifrim D, *et al*. Twenty-four hour ambulatory simultaneous impedance and pH monitoring: a multicenter report of normal values from 60 healthy volunteers. *Am J Gastroenterol* 2004;99:1037–43.
29 Drossman DA. The functional gastrointestinal disorders and the rome III process. *Gastroenterology* 2006;130:1377–90.
30 Savarino E, Zentilin P, Mastracci L, *et al*. Light microscopy is useful to better define NERD and functional heartburn. *Gut* 2014;63:368, 2013–305955.
31 de Bortoli N, Savarino E, Furnari M, *et al*. 657 use of a non-invasive pepsin diagnostic test to detect GERD: correlation with MII-pH evaluation in a series of suspected NERD patients. A pilot study. *Gastroenterology* 2013;144: S–118.
32 Emerenziani S, Cicala M, Zhang X, *et al*. Effect of oesophagitis on proximal extent of gastro-oesophageal reflux. *Neurogastroenterol Motil* 2007;19:459–64.
33 Fiorucci S, Distrutti E, Di Matteo F, *et al*. Circadian variations in gastric acid and pepsin secretion and intragastric bile acid in patients with reflux esophagitis and in healthy controls. *Am J Gastroenterol* 1995;90:270–6.
34 Elabiad MT, Zhang J. Detection of pepsinogen in the neonatal lung and stomach by immunohistochemistry. *J Pediatr Gastroenterol Nutr* 2011;53:401–3.
35 Vakil N. The initial diagnosis of GERD. *Best Pract Res Clin Gastroenterol* 2013;27:365–71.