Original Article

Pepsin as a biomarker of reflux in patients presenting at a UK ENT voice clinic

Peter W. Dettmar[1], Katie H. A. Boulton[1], Andrew D. Woodcock[1], Rhianna K. Lenham[1], Mark Watson[2]

[1]RD Biomed Limited, Castle Hill Hospital, Cottingham, HU16 5JQ, UK; [2]Doncaster Royal Infirmary, Armthorpe Road, Doncaster, DN2 5LT, UK

Contributions: (I) Conception and design: PW Dettmar, M Watson; (II) Administrative support: KHA Boulton, RK Lenham; (III) Provision of study materials or patients: M Watson; (IV) Collection and assembly of data: AD Woodcock; (V) Data analysis and interpretation: KHA Boulton, RK Lenham; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Prof. Peter W. Dettmar (ORCID: 0000-0003-2931-5025). RD Biomed Limited, Daisy Building, Castle Hill Hospital, Castle Road, Cottingham, East Yorkshire, HU16 5JQ, UK. Email: peter.dettmar@rdbiomed.com.

Background: Gastro-esophageal reflux disease (GERD) is common and experienced by 40% of the population. Laryngopharyngeal reflux (LPR) is caused by gastric contents refluxing above the upper esophageal sphincter (UES). LPR diagnosis remains challenging. The aim of this study was to evaluate pepsin as a biomarker of reflux in symptomatic patients presenting at a secondary referral ENT voice clinic.

Methods: All 331 patients and a group of 22 asymptomatic control subjects were recruited. Participants were instructed to provide three saliva samples. Patients provided samples on waking, post-prandial or post-symptom by an episodic or continuous symptom procedure. Saliva samples were analysed for the presence of pepsin using the lateral flow diagnostic device Peptest and the concentration of pepsin in ng/mL determined using the PepCube reader.

Results: The 331 patients produced a total of 982 saliva samples with pepsin detected in 223 (67%) of patients. The greatest pepsin concentration was seen in the post-prandial samples (107.7 ng/mL) however, there was no significant difference between times of sample collection. Control subjects tested negative for pepsin. There was no significant difference in salivary pepsin concentration between genders and no difference in salivary pepsin concentration between patients presenting with episodic (97.5 ng/mL) or continuous symptoms (95.8 ng/mL). The majority of patients were aged between 51 to 80 years with older age tending to correlate with higher salivary pepsin concentrations.

Conclusions: Pepsin was a good biomarker for detecting salivary pepsin in a large population of ENT patients and Peptest under clinical conditions was a reliable and rapid non-invasive diagnostic test.

Keywords: Voice clinic; diagnostic test; laryngopharyngeal reflux (LPR); saliva sample collection; pepsin analysis

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Introduction

Gastro-esophageal reflux disease (GERD) is a prevalent clinical condition in the general population (1). GERD is characterised by an excessive reflux of gastric contents including acid, pepsin and duodenal contents, causing damage and troublesome symptoms (2). These symptoms include heartburn, regurgitation and acid reflux, experienced by around 40% to 60% of the population (3).

The effect of duodenal and gastric contents refluxing into the upper gastrointestinal tract, above the upper esophageal sphincter (UES) causes a condition recognised as Laryngopharyngeal reflux (LPR) (4). LPR is related to reactivated pepsin, examples of symptoms relating to LPR are sore throat, hoarseness, coughing and a globus sensation (5-8). It is believed clinics are seeing 55% of LPR patients experiencing hoarseness (5).

Pepsin present in gastric juice should only be located in the stomach but is found to be present in the saliva of patients

with LPR (9). More recently, LPR has developed a growing interest as a risk factor for laryngeal cancer (10).

Currently LPR diagnosis remains challenging, due to the lack of a 'gold-standard.' Studies have shown the methods for diagnosis often used are a double probe pH monitor and more recently multichannel intraluminal impedance-pH probe monitor (MII-pH) to detect LPR. However, these methods both remain time-consuming, invasive and costly (11-13). The presence of pepsin in a patient's saliva has been identified and confirmed as proximal reflux. This awareness makes pepsin a biomarker of reflux and an underlying pathophysiology of LPR (11).

The need for a non-invasive, novel, and rapid diagnostic method for LPR, using pepsin as a biomarker led to the development of Peptest (RD Biomed Limited, UK), a lateral flow device able to detect and measure pepsin concentration in saliva.

This study aimed to validate the use of Peptest in the diagnosis of LPR in clinical use.

We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/aoe-20-44).

Methods

Recruitment

The patients recruited into this study were from a cohort of patients attending the ENT Voice Clinic. Inclusion criteria: Patients with LPR like symptoms who had a clinical diagnosis of LPR with Peptest introduced to improve diagnostic accuracy and to help prevent misdiagnosis. Exclusion criteria: No clinical diagnosis of LPR. In total 335 patients were consented and enrolled into the study with only four patients failing to provide demographical information, therefore the final study analysis was performed on 331 patients (223 females and 108 males). The mean age was 57 years (range, 16–87 years).

All patients in the study provided samples with either Episodic or Continuous symptoms. Episodic samples defined as occasional heartburn and regurgitation (GERD) or continuous symptoms defined as chronic cough, shortness of breath, hoarseness, lump in throat [extra esophageal reflux (EER) and LPR].

A small number of control subjects (n=22, 6 males,16 females) were recruited from the ENT Voice Clinic at Doncaster Royal Infirmary. This group of subjects had a mean age of 54 years (range 20 to 74 years) were completely asymptomatic with no signs of extra-esophageal reflux disease, heartburn, or regurgitation.

Sample collection

Participants were instructed to provide three saliva samples, the first on waking prior to eating and cleaning their teeth with the patients and control subjects following a different protocol to collect the remaining two samples. Control subjects provided samples post-prandial (1 hour after consuming food) whereas the patients produced samples following either the episodic symptom route, providing samples within 15 minutes of experiencing reflux symptoms on two separate occasions or patients followed the continuous symptom route and provided two samples 1 hour after consuming food on two separate occasions. Participants were informed not to take any medication to treat reflux 48 hours before providing their samples.

All samples were collected into a 30 mL collection tube containing 0.5 mL, 0.01 M citric acid and stored at 4 ℃ prior to pepsin analysis.

Sample analysis

All collection tubes were centrifuged at 4,000 rpm for 5 minutes until a clear supernatant layer was visible. If the supernatant layer was not visible the samples were centrifuged again, and 80 µL from the surface layer of the supernatant sample was drawn up into an automated pipette. The 80 µL sample was transferred to a micro-centrifuge tube containing 240 µL of migration buffer solution (pH 8.2). This sample was vortex mixed for 10 seconds. A second pipette was used to transfer 80 µL of the sample to the circular well of a lateral flow device (LFD) (*Figure 1*) containing two unique human monoclonal antibodies; one to detect and the other to capture pepsin in the saliva samples (Peptest, RD Biomed Limited, UK).

Fifteen minutes after introducing the clinical sample for pepsin analysis into the well, the Peptest LFD was placed into the PepCube reader to determine the intensity of the pepsin test line and the concentration of pepsin in ng/mL in the clinical sample. The developer and manufacturer of Peptest (RD Biomed Limited, UK) determined the lowest detectable level of pepsin for the determination of LPR to be ≥25 ng/mL.

Statistical analysis

All participant data were anonymised prior to the

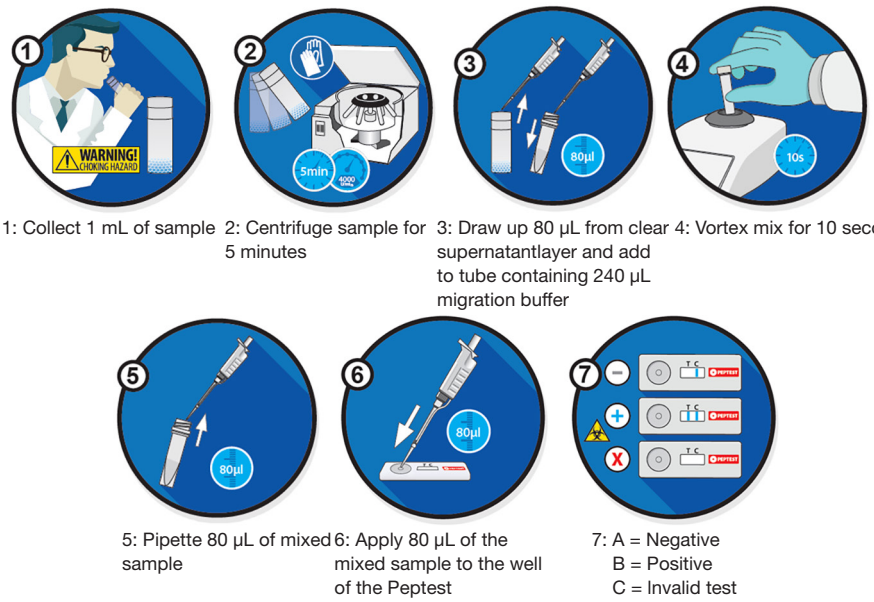

Figure 1 Schematic process for the collection and analysis of saliva samples for the use of Peptest.

completion of this study and the analysis performed. Unpaired t-tests were completed on pepsin concentration (ng/mL) between each sample collection time point, age group and patient subgroup using the statistical package GraphPad Prism 8.2.0 (GraphPad Software, San Diego, CA 92018, USA). The pepsin concentration was used to determine LPR diagnosis. P values <0.05 were considered statistically significant and the mean displayed as±SEM. The sensitivity and specificity were calculated for Peptest.

Ethical statement

This study is a retrospective study and conducted in patients attending a routine outpatient clinic. These were not patients recruited to take part in a clinical trial. Therefore, the ethical approval of this study was exempted by Doncaster Royal Infirmary. Informed consent was obtained from all the individual study participants. All participant data were anonymized prior to the final analysis of the data.

Results

A total of 331 patients and 22 control subjects were recruited and their pepsin analysed in the study. All participants were grouped into subcategories based on their gender. The female/male ratio for patients is 2.06:1 and 2.67:1 for the control subjects. The males had a mean age of 59 years (range, 17–86 years) and females a mean age of 55 years (range, 16–87 years). The control subjects (mean age 54 years; range, 20–74 years) were age matched with the patients recruited into the study.

From the 331 recruited patients a total of 982 samples were analysed for salivary pepsin using Peptest. Seven patients only supplied two saliva samples and two patients only supplied one sample. The greatest concentration of positive pepsin samples was present in the post-prandial sample collection (mean: 107.7±8.4 ng/mL) with the lowest pepsin concentration seen in the on waking samples (mean: 89.6±8.1 ng/mL; *Figure 2*). No significant differences were observed between the samples. All control subjects tested negative for pepsin generating results of 0 ng/mL in all samples.

A comparison by gender was conducted, further breaking down the sample distribution of patients. It was clear that when providing samples on waking or after consuming food, males have a greater concentration of pepsin (*Figure 3*), however the males and females were not significantly different for each sample collection procedure.

The patients were split into groups based on their age range (*Table 1*). It was observed that the youngest age category (≤30 years) had an overall lower percentage of positive Peptest samples and an overall higher percentage of negative Peptest samples compared to the highest age range (≥81 years). However, these observed differences were not significant,

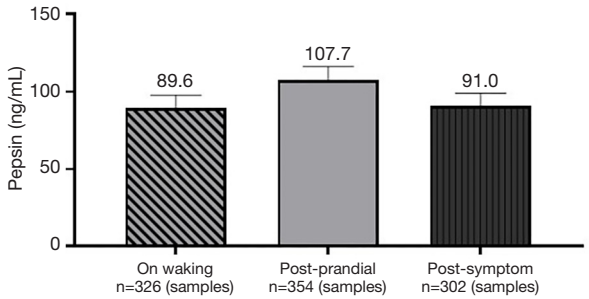

Figure 2 Recorded pepsin concentrations for all samples in each collection category, displaying the mean value, where n = number of samples. No significant difference between each collection category.

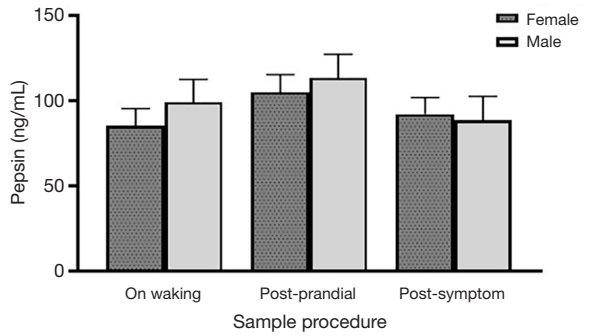

Figure 3 Breakdown of pepsin concentration recorded in each time point for males and females. No significant differences observed.

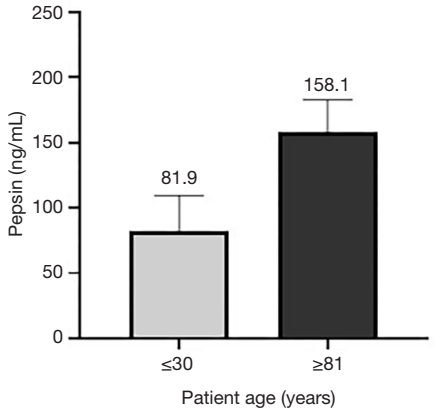

Figure 4 Comparison of pepsin concentration for different age groups. A significant difference of P=0.0441 was observed.

Table 1 Breakdown of patient samples in respect to their age group

Age range, years	Total number of samples	% of samples	
		Pepsin positive	Pepsin negative
≤30	30	50	50
31–40	60	45	55
41–50	131	75	25
51–60	252	74	26
61–70	228	56	44
71–80	240	69	31
≥81	41	86	14

except between the age ranges of ≤30 and ≥81. *Figure 4* displays the younger patients (mean: 81.9±27.6 ng/mL pepsin) involved in this study compared to the eldest participants (mean: 158.1±24.6 ng/mL pepsin). A significant difference was observed between these age ranges with a P value of P=0.0441. The data presented in *Table 1* suggests the diagnosis of reflux disease tends to occur in middle aged patients as 73% of samples were provided by patients aged 51–80 years.

Due to LPR often being referred to as silent reflux, patients were split into categories (*Figure 5*) based on their symptoms and sample collection procedure (episodic or continuous). On examination of the results, those patients categorised as episodic had a slightly higher but not significant (P=0.8581) concentration of pepsin (97.5±6.9 ng/mL) compared to patients categorised as presenting with continuous symptoms (95.8±6.5 ng/mL).

Discussion

At least twenty five percent of patients presenting with upper gastrointestinal symptoms will have some symptoms of LPR (14) and in a study by Kamani *et al.* (15) they postulated that the prevalence of LPR symptoms in the UK population was as high as 34.4%. However, a definitive clinical diagnosis of LPR is never that straight forward and can be challenging. This was particularly difficult in the current study investigating patients in a single ENT clinic who previously had a failed clinical diagnosis of LPR. Peptest was introduced into the clinic to improve the diagnostic accuracy and to help prevent misdiagnosis amongst this group of patients.

The mean age range of the patient group was 57 years with the majority of patients aged between 51 and 80 years of age (73%). However, the patients with the highest level

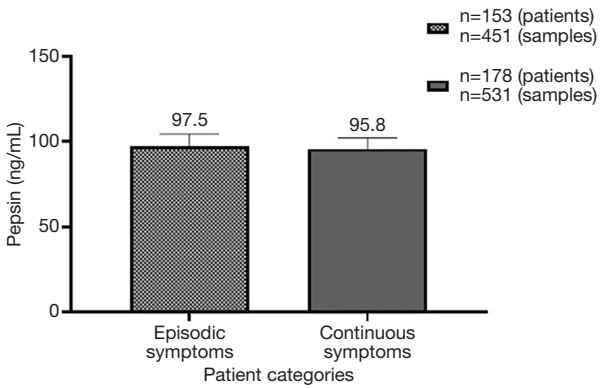

Figure 5 A comparison of mean pepsin concentration for patients presenting with Episodic or Continuous symptoms. No significant difference was observed.

of pepsin were those over 80 years of age with a pepsin concentration of 158.1±24.6 ng/mL compared with a pepsin concentration of 81.9±27.55 ng/mL in the younger group, significant at $P<0.05$. This result is not dissimilar to that reported by Thompson *et al.* (16) who found in their study that the elderly and middle aged groups of patients had statistically significantly higher symptoms of dyspepsia than the younger age groups. Age is an important element to consider in LPR pathology and was recently demonstrated by Gelardi *et al.* [2017] (17). Their findings showed a significant correlation between the increase in the patients age and the pepsin increase within the esophagus. This was further noted in a systemic analysis on the prevalence of GERD (18). This study, however, did not measure pepsin concentration in their patient groups.

Three hundred and thirty-one patients were recruited in to the current study, many of whom had challenging symptoms for a long time and had previously been seen in primary care and alternative secondary care clinics such as gastroenterology, but symptoms persisted following failed treatments. Some patients had previously had double probe 24-hour pH monitoring and in some cases multichannel intraluminal impedance (19). These diagnostic tests are invasive and time consuming and expensive especially in the UK and not available or suitable for all patients (20,21). One of the major aims of this study was to validate a non-invasive and highly patient compliant diagnostic test, which is both rapid and cost effective. Such a test also needed to be reliable, sensitive, and specific. The patient group was compared directly with an asymptomatic age matched control group who had never experienced upper gastrointestinal symptoms of heartburn, regurgitation or reflux or had any family history of upper gastrointestinal disease. One criticism of the current study is the size of the age matched control group which would have benefited from being larger. However, we were able to show a clear distinction in terms of the presence of salivary pepsin between patients presenting with suspected symptoms of LPR and asymptomatic controls.

There were more female patients than male patients (223:108) with males found to have higher but not significant pepsin levels both on waking and post-prandial. In a study with reasonably high level of patients we can conclude that gender does not influence pepsin concentration. The majority of patients provided three saliva samples and clearly pepsin concentration was higher in the post-prandial sample.

We directly compared patients based on their symptom profiles and their saliva sample collection category. Patients fell into one of two profiles either presenting with episodic symptoms or continuous symptoms. One hundred and fifty-three patients presented with an episodic symptom profile and these patients were categorised as having classical GERD like symptoms for example heartburn and regurgitation as well as LPR symptoms. The mean pepsin profile in this group of patients was 97.5±6.9 ng/mL. The one hundred and eighty-six patients categorised with continuous symptoms were those patients presenting solely with LPR symptoms and with a mean pepsin concentration of 95.8±6.5 ng/mL. There was no difference in the mean pepsin concentration between patients presenting with episodic or continuous symptoms.

There are several limitations of our study including the size of the asymptomatic control group, which was age matched but overall, the number of control subjects was low. The study would have benefited from recruiting a higher number of control subjects. Having information on patient history, lifestyle and especially information on diet, history of smoking and alcohol use would have been advantageous to the study. Alcohol especially may be considered a risk factor of reflux (22). The study would also have benefited from more patients being tested with impedance-pH monitoring allowing a direct comparison with salivary pepsin analysis. Lastly, one major limitation was the fact that there is no truly recognised 'gold standard' test available for diagnosing LPR which leaves the detection of pepsin as a biomarker for determining whether LPR is present or not present as the potential LPR diagnostic of the future.

Finally the detection of pepsin has been previously reported and established as a reliable biomarker for the diagnosis of LPR (23-29). In the current study of the 331 patients recruited 223 were analysed as pepsin positive by Peptest. In this study the control subjects had three negative pepsin samples, generating a pepsin concentration of 0 ng/mL. The final analysis for the total LPR patient population and the relatively small number of asymptomatic control subjects showed a sensitivity of 68% and a specificity of 100%.

Peptest has the potential to provide a low cost, rapid and easy to use solution for diagnosing reflux disease. It is non-invasive and can be used with good patient compliance across the complete age spectrum.

Through the adoption of the Peptest technology patients can provide saliva samples from the comfort of their own homes. Saliva samples can be posted to their health care provider or directly to the central testing laboratory for analysis. Pepsin analysis reports are available within 20 minutes of patients providing their saliva samples and a report is sent directly to the patient and their health care provider.

Peptest has been fully validated in two large clinical studies. The first in the UK in 1,031 ENT patients and control subjects (30) and the second in 1,032 gastroenterology patients and control subjects from China (31). Both studies demonstrated sensitivity and specificity comparable or superior to currently used invasive diagnostic tests (32). The present study showed the value of using pepsin as a biomarker for identifying patients presenting with reflux disease.

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Footnote

Provenance and Peer Review: This article was commissioned by the editorial office for the series "Epidemiology, Biomarkers and Modelling of Gastroesophageal Reflux Disease" published in *Annals of Esophagus*. The article was sent for external peer review organized by the editorial office.

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/aoe-20-44). The series "Epidemiology, Biomarkers and Modelling of Gastroesophageal Reflux Disease" was commissioned by the editorial office without any funding or sponsorship. PWD served as the unpaid Guest Editor of the series and serves as an unpaid editorial board member of *Annals of Esophagus* from Mar. 2020 to Feb. 2022. PWD is a Director of RD Biomed LTD. ADW and RKL report they are Employed by RD Biomed LTD. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study is a retrospective study and conducted in patients attending a routine outpatient clinic. These were not patients recruited to take part in a clinical trial. Therefore, the ethical approval of this study was exempted by Doncaster Royal Infirmary. Informed consent was obtained from all individual study participants. All participant data were anonymised prior to the final analysis of the data.

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