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Studies of Salivary Pepsin in Patients with GERD

Short title: Salivary Pepsin in GERD

Caroline Race[1,5] Joanna Chowdry[2,5] Jean M. Russell[3,] Bernard M. Corfe[2,4,6] & Stuart A. Riley[1,4,6,7]

1. Department of Gastroenterology, Northern General Hospital, Herries Road, Sheffield
2. Molecular Gastroenterology Research Group, Academic Unit of Surgical Oncology, Department of Oncology and Metabolism, University of Sheffield
3. Department of Corporate Information and Computer Services, University of Sheffield, Hownslow Road, Sheffield
4. Insigneo Institute, University of Sheffield
5. These authors made an equal contribution to this work
6. These authors made an equal contribution to this work
7. *Author for correspondence*: Dr Stuart Riley

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SUMMARY

Background

Gastro-oesophageal reflux disease (GERD) is difficult to diagnose without invasive testing. Peptest is a newly marketed diagnostic tool which aims to quantify salivary pepsin as a marker of reflux, providing a rapid alternative to invasive procedures. We aimed to evaluate optimal timing for sampling and to evaluate the accuracy of Peptest against an independent measure.

Methods

Thirty diagnosed GERD patients (12 female, mean age 49 (range 20-72)) and twenty asymptomatic subjects (14 female, mean age 56 (range 21-56)) were subject to diurnal saliva sampling, with additional samples for sixty minutes following self-reported reflux symptoms and following triggering of a proximal reflux alarm. Saliva samples were split and were analysed by both Peptest and ELISA with operators for each blinded to sample identity.

Results

Salivary pepsin was detectable in most patients and most volunteers. Peptest scores were significantly lower for patients than controls (P<0.005). ELISA scores showed no difference between patients and controls. There was no effect of diurnal sampling time (P=0.75) or time after symptoms (P=0.76) on Peptest readout. There was no correlation between Peptest and Pepsin ELISA (P=0.55); Bland-Altman analysis suggested no agreement between the tests (P=0.414) implying that they do not measure the same analyte. Receiver-operator curve suggests that neither Peptest (P=0.3328) nor pepsin (P=0.4476) is useful for predicting GERD .

Conclusions

Salivary pepsin is not a reliable tool for the diagnosis of GERD.

198 words

INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most common gastrointestinal disorders and is defined as a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications [1]. Although the typical symptoms that accompany reflux into the oesophagus are heartburn and acid regurgitation, non-specific symptoms are not uncommon [1]. Furthermore, when gastric refluxate passes the upper esophageal sphincter (EOR) a range of laryngeal, pharyngeal and respiratory symptoms including cough and recurrent infections may result [2].

Current diagnostic methods for GERD include symptomatic assessment (including structured questionnaires [3], therapeutic trials with proton pump inhibitors [4,5], endoscopy [6] and ambulatory esophageal reflux monitoring [7]). Unfortunately, all have limited sensitivity and specificity [6] furthermore the latter two are invasive. There is therefore an unmet need for non-invasive diagnostic tools with good sensitivity and specificity for this condition.

Pepsin is a protease which is synthesised, via its precursor pepsinogen, in gastric chief cells [8] and its presence in the oesophagus or more proximal sites is argued to be indicative of reflux [9]. Interestingly pepsin has been reported in laryngeal and nasal sinus tissues, tracheal secretions and broncho-alveolar lavage fluid and in saliva [9].

Recent reports have suggested that salivary and/or sputum pepsin may be a clinically useful, non-invasive diagnostic marker for GERD. A commercially developed lateral flow test device for detecting and quantifying pepsin as a biomarker of reflux in saliva and sputum samples has been released. Although no definitive evaluation is available in the peer-reviewed literature, the five studies using it to date have yielded conflicting results [10-14] and NICE remains cautious about endorsing its use. Peptest comes with little information on the optimal timing for sampling, persistence of pepsin in the mouth following reflux, or indicative limits of detection, or discriminatory potential from the normal population. The aim of this study was to assess the best time to take samples for salivary pepsin assay and to validate the findings against both conventional reflux testing and against a validatable pepsin assay in both GERD patients and normal volunteers.

METHODS

Study Design

This was a case-control comparison of a commercial diagnostic assay. Cases were symptomatic patients referred for anti-reflux surgery, controls were asymptomatic volunteers. The study had two parts: (i) an initial pilot assessed feasibility of diurnal repeated saliva sampling, collection and posting on the day of return by the patient, all following manufacturer recommendations, in 10 cases and 10 controls; (ii) an extended study using the same sampling and storage protocol in a further 20 cases and 10 controls, with samples split and also analysed by a second independent method.

Subjects

Studies were performed on asymptomatic healthy volunteers and patients referred for consideration of anti-reflux surgery. Healthy volunteers were recruited by advertisements placed in Sheffield Teaching Hospital NHS Trusts. Volunteers with a history of upper gastrointestinal symptoms or those taking medications known to influence upper gastrointestinal function were excluded. Patients were recruited from referrals to the Gastrointestinal Physiology Unit for esophageal reflux and motility testing prior to consideration of a surgical antireflux procedure. All had typical reflux symptoms and had had a good or partial response to proton pump inhibitor treatment. Patients known to have other non-reflux related upper gastrointestinal disease or general disorders or drugs (other than acid suppression) known to influence upper gastrointestinal function were excluded. The study was approved by the Sheffield Teaching Hospital NHS Ethics Committee (NRES Committee Yorkshire & Humber 12/ YH/0466) and Sheffield Research Department (STH16373).

Study Protocol

Following written informed consent all subjects underwent a detailed symptom assessment and drug history and were asked to complete two validated symptom questionnaires which have a high sensitivity for reflux disease (the Gastro-esophageal Reflux Score Questionnaire (GORS) [3] and the Hull Reflux Cough Questionnaire (HARQ) [15]. Selection was based on positive GORS (≥4) with HARQ (≥13) included for broader assessment of symptoms. Clinical discussion occurred on the day to confirm the symptoms described by patients agreed with scores before inclusion. All participants were studied off proton pump inhibitor therapy for at least 7 days (pharmacokinetic analysis of PPI suggests that clearance occurs with a 60 minutes half-life, and furthermore that PPs have a half-life of 56hr and a daily turnover rate of 20%, supporting 7 days as an adequate wash-out window[16] in line with previous

assessments of Peptest[17]), H_2RAs for 2 days and over the counter antacids and alginates for 24 hours.

Subjects were asked to fast for 4 hours prior to study and then underwent high-resolution esophageal manometry and 24-hour dual pH testing and produced saliva samples for pepsin analysis as detailed below.

High resolution manometry was performed using Manoscan equipment (Sierra Scientific Instruments, Los Angeles USA). Esophageal pH testing was performed using a dual channel pH catheter (Versaflex®Given Imaging, Mansfield USA) attached to a novel data logger/ alarm device (PDTronics, Sheffield, UK). Since acid reflux episodes are often asymptomatic we developed a device to fulfil the normal functions of a pH datalogger but also produce an audible and visible alarm when the proximal pH channel fell below pH 4. This prompted the subjects to start collecting saliva samples following the first detected proximal acid reflux events. Following interim analysis of the 10 volunteers and 10 patients, esophageal impedance was additionally performed using the Sandhill system (Sandhill Scientific (Diversatek Healthcare, Milwalkee USA). Prior to the 24 hour study, pH catheters were placed such that the sensors were 5 and 20 cm above the manometrically determined lower esophageal sphincter (LOS) and the six impedance sensors were placed at 3, 5, 7, 9, 15 and 17 cm above the LOS. All subjects were asked to record symptoms, meal times and periods of recumbency.

All tracings were reviewed manually to ensure accurate reflux detection. Proximal reflux was defined as refluxate reaching the 20 cm pH and or 17-cm impedance sensor. Symptom index (SI) and Symptom Association Probability (SAP) were used to characterise the association between symptoms and reflux.

Saliva sample collection and analysis

In order to identify the optimal timing of saliva sampling for pepsin analysis all subjects were asked to collect saliva samples; 2, 5, 10, 15, 30 and 60 minutes after reflux symptoms and after activation of the proximal reflux alarm. Since reflux is common after meals and periods of recumbency subjects were also asked to collect similarly timed saliva samples on rising in the morning (before eating, drinking or teeth brushing) and after the evening meal. Saliva was collected following RD Biomed sampling and packaging instructions and using their buffer (0.5 ml of 0.01 M citric acid) and were stored as recommended (4°C) and returned at the end of the 24-hour study period on the same working day (as per RD Biomed sampling instructions). All samples were coded and analyses were performed blind to all clinical and physiological variables. For the extended study all salivary samples were homogenised by vortex mixer and split in two, one half sent for Peptest analysis (Peptest, RD Biomed, Hull), the second half was quantified for pepsin by ELISA at The University of Sheffield. Samples

received on ice at The University of Sheffield were frozen immediately and stored at -80°C prior to assay.

Peptest assay All salivary samples were sent to RD Biomed for proprietary analysis of pepsin (Peptest, RD Biomed, Hull) which was undertaken as paid service provision (i.e. the samples were presented as for diagnosis, not part of a research collaboration). Quantification of pepsin by Peptest was provided by RD Biomed as a data sheet via email usually with 1-2 working days of the sample being sent.

ELISA pepsin assay was an indirect ELISA using antiPepsin A monoclonal antibody (Santa Cruz sc-365680, raised against an antigen of aa281-324 of Pepsin A). The ELISA was calibrated using recombinant human pepsin A (Stratech SCB-RP112894h, which includes aa15-388 of Pepsin A). The ELISA was linear when tested in the range from 0-150ng with a limit of detection of 2ng. For development and validation of the ELISA see Supplementary Online Information (SOI, Section 1).

Statistical analyses

Statistics were undertaken as indicated using SPSS 23 (IBM, Armonk, New York, 2015), other than Bland and Altman Tests, which were produced using R (3.4.1), with the packages BlandAltmanLeh (v 0.3.1) and ggplot2 (v2.2.1).

RESULTS

Subjects and demographics

Control group: 50 volunteers responded to the advert; 20 (6 male, 14 female, aged 21 to 56 years) met the inclusion criteria and completed the study, 3 had exclusion criteria, 26 declined intubation and one was unable to tolerate intubation

Patient group: 101 patients were approached; 30 (18 male, 12 female, aged 20 to 72 years) met the inclusion criteria and completed the study, 49 declined to participate, 13 were excluded as they did not meet the symptom criteria, 8 were excluded as the pH study was negative at the time of processing the salivary samples and one was unable to tolerate the intubations. All the volunteers denied reflux symptoms and all 30 patients reported regular heartburn and or regurgitation. Questionnaire scores were significantly different between the two groups (GORS: volunteers 0(0-0) versus patients 10(3-15) $p < 0.005$; HARQ: volunteers 0(0-9) versus patients 28 (1-65) ($p< 0.005$).

All 20 volunteers had esophageal acid exposure times within the normal range (median 1.0%, range 0 - 3.5%). The 10 volunteers who also underwent MII had values for non-acid and gas reflux within the normal range (mean total number of reflux events 14.6). Of the 30 patients studied 23 had esophageal acid exposure times in excess of the normal range (Median 8.85, and range 3.0 (one individual with significant supine reflux) – 39%) and were classified as having true acid reflux. A further 3 patients had normal acid exposures (Median 2.85, range 2.6-4.6%) but had abnormal non-acidic reflux (Median 81, range 74-101 events). Four patients were found to have normal reflux values on the day of study and all demonstrated a negative SAP and were therefore deemed to have functional heartburn and were excluded. A positive SAP was classified as >95%.

In patients with true acid reflux, 20 reported symptoms during the 24-hour period and in 12 the proximal reflux alarm was triggered. Manual analysis of the dual pH tracings confirmed that such episodes were all associated with both distal and proximal esophageal acidification.

Salivary Pepsin analysis

In the initial pilot study, the ten volunteers produced a total of 138 salivary samples for Peptest analysis. Seven of the volunteers had detectable levels at reported concentrations of 25 – 250 ng/ml, often in multiple samples. Of the seven patients who had true reflux, all had detectable levels at concentrations of 25 – 250 ng/ml in at least one and often in several samples. The lack of a clear difference between asymptomatic volunteers and patients

prompted us to investigate the validity of the Peptest. We therefore extended the study to a further 10 volunteers and 20 patients following an identical protocol but splitting saliva samples for analysis by both Peptest and an in-house indirect ELISA.

Peptest analysis of 276 samples from the 20 volunteers revealed detectable levels in 102 samples in total (37%). 75 % of volunteers had at least one positive sample for salivary pepsin (range 1-18 positive samples). Peptest analysis of 458 samples from 25 of the 30 patients with true reflux revealed positive results in 41 % of samples. 84 % of patients had at least one positive result. The indirect ELISA of 141 samples from the 10 volunteers revealed detectable levels in 122 samples (87 %). All 10 (100 %) of volunteers had at least one positive sample for salivary pepsin. Analysis of 393 samples from 20 patients with pathological reflux revealed positive results in 309 (79%) of samples. All 20 (100 %) of patients had at least one positive result.

The mean scores and ranges between the asymptomatic control group and the GERD-diagnosed patients were compared (Fig 1A). Peptest gave a significant difference in score between the control and GERD groups, but the controls gave the higher reading (t-test, $p<0.01$). In contrast, there was no significant difference in the average pepsin concentration in each group by ELISA. There were significant differences between the readouts of the two quantitation methods within groups (Mann-Whitney, $p<0.01$ for Control arm, $p<0.001$ for GERD arm).

To assess whether timing of sampling influenced outcome the data were also analysed according to sampling point (rising, post-prandial, post-alarm, post symptoms), and differences between groups tested. Peptest quantitations (Fig 1B) were not significantly different between patients and volunteers after rising or evening meal, but were significantly lower in the patient group on reflux alarm ($p<0.05$, without Bonferroni correction). A comparison was undertaken between Peptest scores for 2-60 minutes following reflux alarm in controls and patients (Fig1C). The Peptest results showed no difference in salivary readout across time, but symptomatic individuals had lower readouts than the control group ($P<0.05$, one way ANOVA). We assessed the value Peptest as a diagnostic tool using a receiver-operator (ROC) analysis (Figs 1D). Peptest yielded an area-under-the-curve of 0.528 with P=0.333 indicated the test is worthless.

Finally, a linear mixed model [18]was deployed to assess contributions to variance allowing for the repeated nature of measures with respect to time using a First Order autoregressive, Fig 1E. No variable explained significantly any aspect of the variation in data. In summary, the

readouts for Peptest were lower for subjects with diagnosed GERD than for asymptomatic controls when all samples were considered. Furthermore Peptest readouts were not significantly different in either fasted state or following reflux for subjects with GERD than asymptomatic volunteers.

Comparison of Peptest and ELISA analyses

As distinct outcomes were found for the Peptest and ELISA analyses (see section 2) direct comparisons were undertaken between the quantitations from the two analyses. Quantifications were compared in several ways (Fig 2). Quantitations from each method were plotted against each other and their relationship tested using a Spearman's correlation undertaken (Fig 2A). This test did not indicate any statistically detectable relationship between the outcomes from each test.

As the data did not correlate we assessed their distributions independently. The ELISA data followed a biologically usual gamma-distribution (SOI section 2). The Peptest data did not follow a gamma distribution and show large frequencies outside the measurement limits (SOI, Section2): the data were in a range of 0-500ng, but 73% of the points were outside the limits of the sensitivity of measure (63% below and 10% above). To try to identify relationships, data were split into three categories according to Peptest result (0, 500 and 0<datapoint<500). The distributions of ELISA data for each Peptest category were analysed and shown to have no significant difference (SOI, Section 2). Bland-Altman analysis is a standard statistical option for comparing between two tests of the same endpoint, and was used to assess whether the assays were measuring the same substance (Fig 2Bi and ii). The plots shows a cluster of datapoints where both tests are at their minimal point. Fig2Bi presents untransformed data which exhibit poor distribution due to the narrow range. Data were therefore log transformed (Fig 2Bii). Whilst the distribution is wider, the paucity of data in the sensitive range for Peptest distorts the graph. Intraclass Correlations (ICC) were undertaken for both the untransformed and log transformed data. For the untransformed data the ICC is 0.019 (p=0.414) for the transformed data the ICC is 0.54 (p=0.268). These ICC data suggest that the two tests are not measuring the same endpoint. As the ELISA is undertaken with a commercially validated antibody, revalidated against recombinant pepsin, shown to be linear for pepsin, and follows a biologically common gamma distribution, we deduce that it is reliably measuring pepsin. We therefore infer that Peptest is not measuring pepsin.

Evaluation of salivary pepsin and Peptest as a diagnostic markers for GERD

The ELISA quantitations were then independently assessed in terms for potential to predict GERD. When ELISA quantitations were assessed globally, there was no significant

difference between the amount of pepsin diagnosed and asymptomatic individuals (Fig 3A). As for the Peptest analyses, quantitative data for pepsin were stratified by sampling time (rising, postprandially, after alarm and after symptoms). There were no significant differences in salivary pepsin concentration at any of these timepoints (Fig 3B). When pepsin was analysed in the time period following a reflux alarm there were no significant differences between volunteers and patients, and no significant changes across time (Fig 3C). Finally, the data were dichotomised as RD Biomed have suggested a diagnostic threshold of 16ng/ml [17], which demonstrated rates of positives were identical in the GERD and control groups (SOI, Section 3). Levels of pepsin in saliva did not discriminate between asymptomatic subjects and patients diagnosed with GERD in any experimental setting. Finally we assessed the value of the Pepsin ELISA as a diagnostic tool using ROC analysis (Fig 3D). The area-under-the-curve analysis was 0.522 with P=0.448 indicating that the test is worthless.

DISCUSSION

Peptest is a highly promoted diagnostic device presented for assessment of reflux through detection of pepsin. It utilizes a proprietary monoclonal antibody (only available to RD Biomed) in a lateral flow format to quantify levels of pepsin in saliva (based on the working hypothesis that pepsin in saliva is non-routine and can only be attributed to reflux). A recent review of studies assessing salivary pepsin for LPR [19] erred to a conclusion that pepsin may be of value, however studies were inconsistent with multiple methodologies deployed, a range of cut-offs and differences in study design and in control populations. Only one study using Peptest was of sufficient rigour for inclusion in that systematic review [12] reporting that Peptest yielded the same rates of positives in cases and controls of LPR.

Our initial aim was to establish optimal timings for saliva sampling relative to diurnal variation and to measure spiking and persistence of pepsin in saliva following reflux to support optimal application of Peptest. However a very high rate of false positives (and negatives) relative to clinical diagnostic criteria led to re-evaluation of both Peptest and pepsin data in the context of GERD specifically. Our finding is that pepsin is present in control, asymptomatic subjects and at measurable levels and does not have discriminatory potential for separating GERD from controls. This finding is consistent with other reports which found no difference between cases and controls [9,20,21], or found controls had higher levels [22]. A recently published study concluded a value for Peptest in predicting GERD, however there was no difference between controls and GERD patients when all sampling was taking into account, suggesting low reproducibility [10]. A second recent paper assessing application of Peptest in GERD also showed poor reproducibility of Peptest and depended on data-selection to achieve any diagnostic power[17]. Peptest outcomes were provided to us by RD Biomed as a quantification in ng/ml of pepsin in saliva. When analysed, the distribution of these data was very skewed (Fig 2). As the analytical platform is a window in a lateral flow device, there may be very significant potential for subjective score or operator error.

The presence of pepsin in saliva is thought to be indicative of gastric refluxate and is the premise of test development, however recent data arising from the Fantom5 project [23] indicates expression of pepsin in the tongue (SOI, section 3). This outcome from a high-throughput screen requires direct validation but provides a plausible explanation for presence of pepsin in saliva in controls as an apparent inconsistency reported across several studies.

A recent review and guideline advises the use of a constellation of scores in diagnosis of GERD and shifts away from a single measure [24]. Our report demonstrates inconsistencies

between ELISA quantitations (using a validated commercial antipepsin antibody, re-validated and calibrated against pepsin from a different supplier) and Peptest quantitations, and demonstrated an unusual distribution of data for Peptest by two separate statistical tests. Further recent work questions the diagnostic value of Peptest / pepsin[25]. As far as we can determine using a formal statistical test, Peptest and our pepsin ELISA are measuring differing analytes. We suggest that Peptest is neither useful as a diagnostic for GERD, nor is a measure of pepsin.

CONCLUSIONS

- There is an unmet need for non-invasive diagnostics for GERD, salivary pepsin has been proposed as one such test;
- Our data and those of others indicate pepsin does not discriminate effectively between healthy asymptomatic controls and patients with confirmed reflux;
- Our data suggest that Peptest does not reliably measure pepsin.

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CONFLICT OF INTEREST

Guarantor of the article: Dr Stuart Riley

Specific author contributions: SAR conceived the study, directed the clinical aspects, oversaw data analysis and interpretation and revised the drafts; CR undertook the recruitment, sampling and clinical assessments, managed the data and undertook preliminary analysis, contributed to the drafting of the paper; JC undertook ELISA development and validation, quantifications, undertook preliminary analysis and contributed to the drafting of the paper; JMR undertook and directed statistical analyses and interpretation; BMC contributed to experimental design and interpretation, directed the lab analyses and drafted the paper. All authors approved the final submitted version of the paper.

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LEGENDS

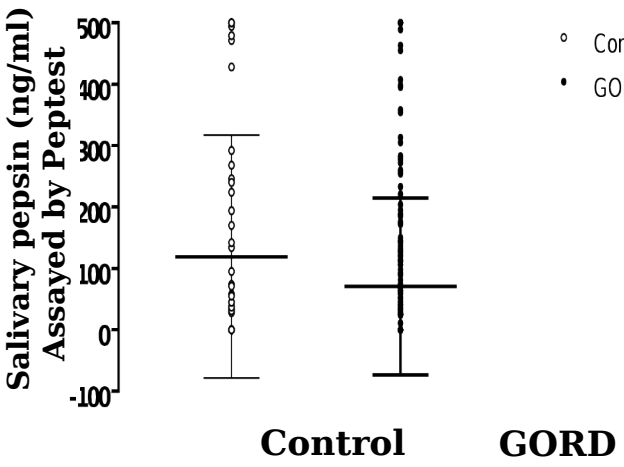
Figure 1. Analysis of discrimination between patients and controls by Peptest.
Panel A shows a comparison of Peptest quantitations for all samples for the controls (open circles) and GERD patients (closed circles). The average in controls was significantly higher than patients ($p<0.005$, unpaired t-test). Panel B compares the mean readout for Peptest, stratified by sampling time and by patients (grey filled bars) or controls (white bars). There was no significant difference between patients and controls at any time except following the reflux alarm, when controls were higher than patients. No controls reported a reflux symptom, so no comparison was undertaken. Panel C shows Peptest readout across the 60 minutes following reflux alarm for controls (open circles) and patients (filled circles). There was no significant difference across time. Patients scores were significantly and consistently lower than controls (P,0.05, 1-way ANOVA). Event rate was very low in the control group with no self-reported symptoms and only three alarms across the study. Panel D Receiver-Operator Curve (ROC) analyses of Peptest data. The dataset had an AUC of 0.53 (P=0.333). Panel E shows variance component analysis using multi-level modelling (Repeated Measures ANOVA) for GERD patients and with regards to event type and sample time using a First Order autoregressive (SPSS 24). The results demonstrate no significance except when sample taken during a reflux event.

Figure 2. Comparison of Peptest and pepsin ELISA.
Panel A shows Spearman's correlation between quantifications of pepsin in Saliva from both Peptest (x-axis) and indirect ELISA (y-axis). Correlation yielded a very weak r^2, of 0.0007, which was not significant. Panel B shows Bland-Altman plots for the same comparison: Panel Bi is untransformed data (Intraclass correlation (ICC)=0.019, P=0.414), Panel Bii is log-transformed data (ICC=0.54, P=0.268).

Figure 3. Pepsin does not predict GERD
Panel A shows a comparison of ELISA pepsin quantitations for all samples for the controls (open circles) and GERD patients (closed circles). There was no difference between groups (unpaired t-test). Note the difference in dynamic range of these data (0-100) by comparison with Peptest scores (Fig1A, range 0-600)
Panel B compares the mean readout for pepsin, stratified by sampling time and by patients (grey filled bars) or controls (white bars). There was no significant difference between patients and controls at any time. No controls reported a reflux symptom, so no comparison was undertaken. Panel C shows pepsin ELISA readout across the 60 minutes following reflux alarm for controls (open circles) and patients (filled circles). There was no significant

difference across time (1-way ANOVA). Panel D Receiver-Operator Curve (ROC) analyses of pepsin ELISA data. The dataset had an AUC of 0.52 (P=0.448).

A



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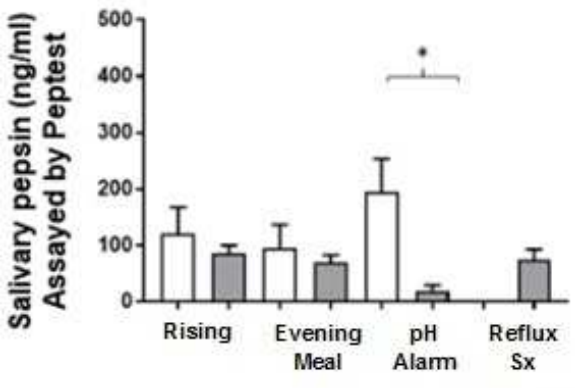
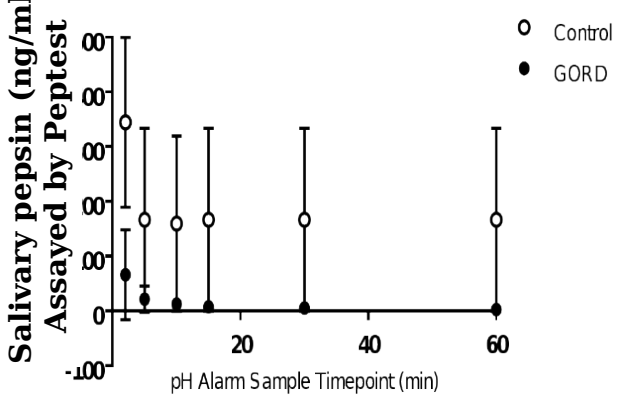
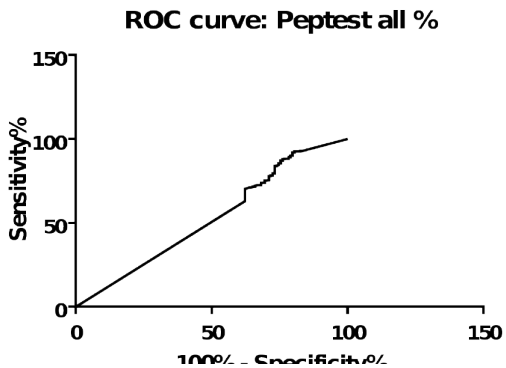


C



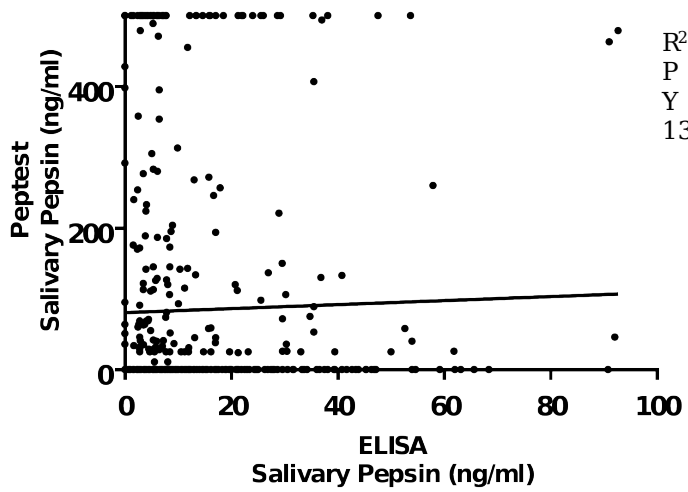
D



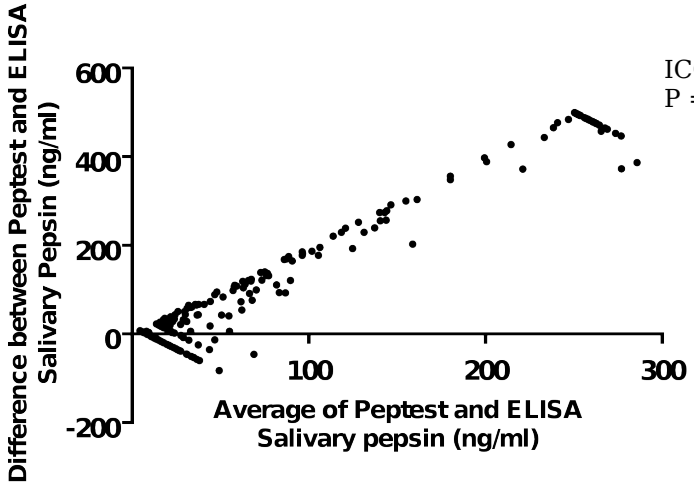
E

Source	Numerator Df	Denominator df	F	Sig.
Event Type	3	51.91	0.424	0.736
Sample Time	5	208.13	2.668	**0.023**
Event Type * Sample Time	15	207.95	1.184	0.286
GORD Diagnosis	1	34.74	0.036	0.851
Event Type * GORD Diagnosis	3	51.91	0.324	0.808
Sample Time * GORD Diagnosis	5	208.131	0.466	0.801

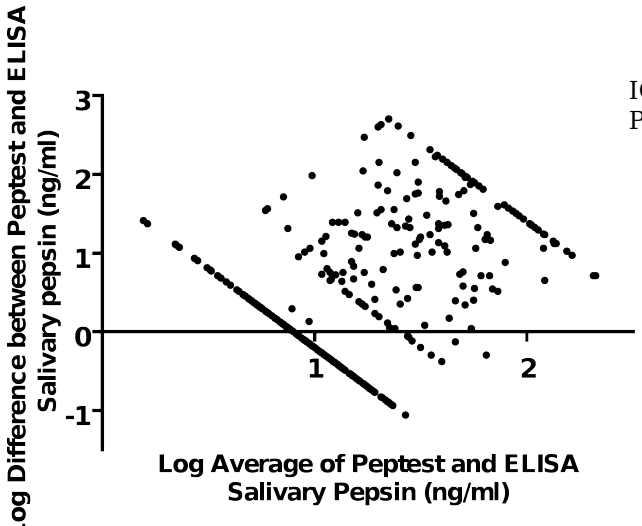
A

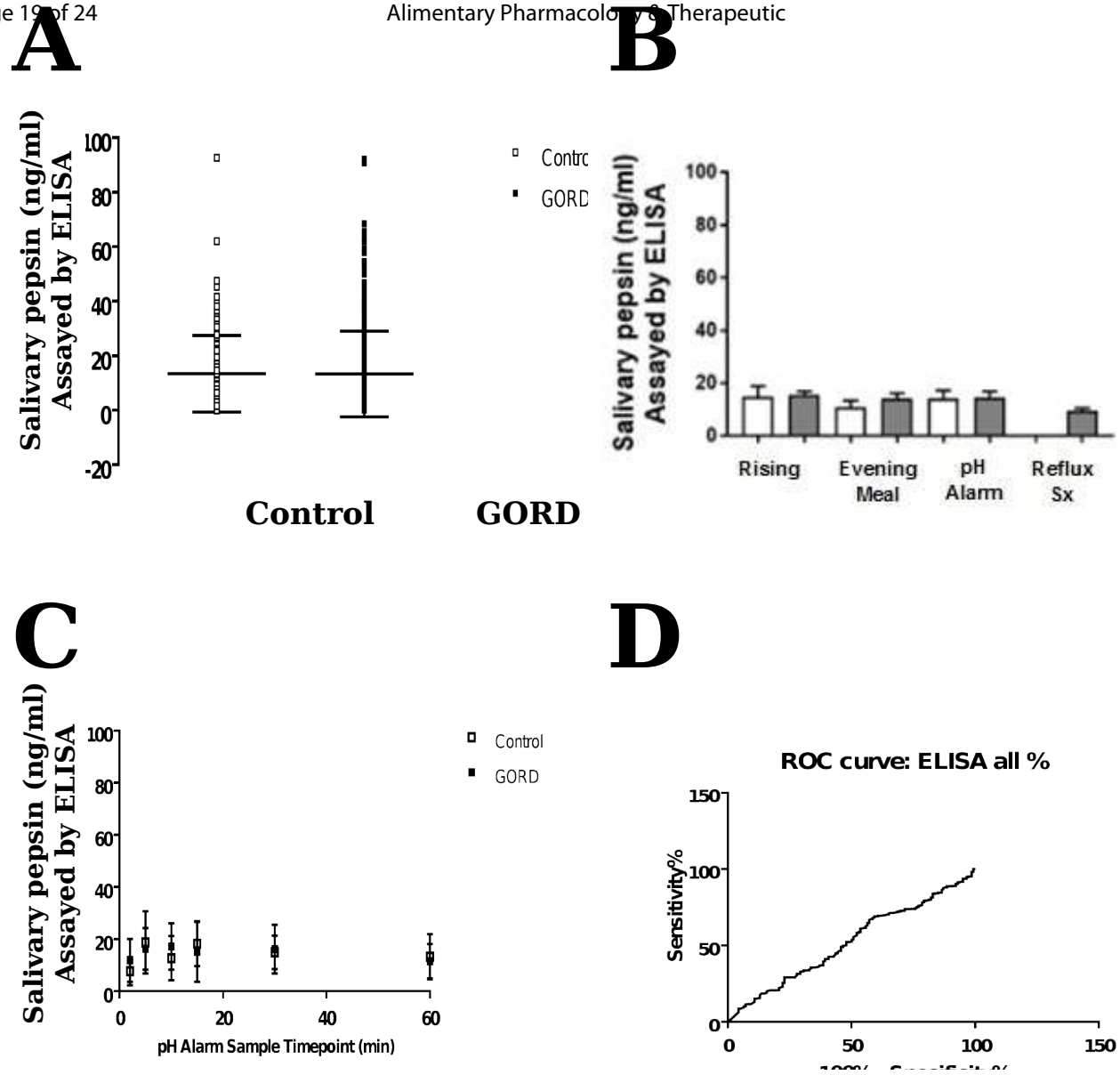

Peptest Salivary Pepsin (ng/ml)
400
200
0
0
20
40
60
80
100
ELISA Salivary Pepsin (ng/ml)
R² = 0.0007
P = 0.55
Y = 0.0025x + 13.05

Bi


Difference between Peptest and ELISA Salivary Pepsin (ng/ml)
600
400
200
0
-200
100
200
300
Average of Peptest and ELISA Salivary pepsin (ng/ml)
ICC = 0.019
P = 0.414

ii


Log Difference between Peptest and ELISA Salivary pepsin (ng/ml)
3
2
1
0
-1
1
2
Log Average of Peptest and ELISA Salivary Pepsin (ng/ml)
ICC = 0.54
P = 0.268


A
Salivary pepsin (ng/ml) Assayed by ELISA
100
80
60
40
20
0
-20
Control
GORD
B
Contro
GORD
Salivary pepsin (ng/ml) Assayed by ELISA
100
80
60
40
20
0
Rising
Evening Meal
pH Alarm
Reflux Sx
C
Salivary pepsin (ng/ml) Assayed by ELISA
100
80
60
40
20
0
0
20
40
60
pH Alarm Sample Timepoint (min)
Control
GORD
D
ROC curve: ELISA all %
Sensitivity%
150
100
50
0
0
50
100
150

Supplementary Online File

for:

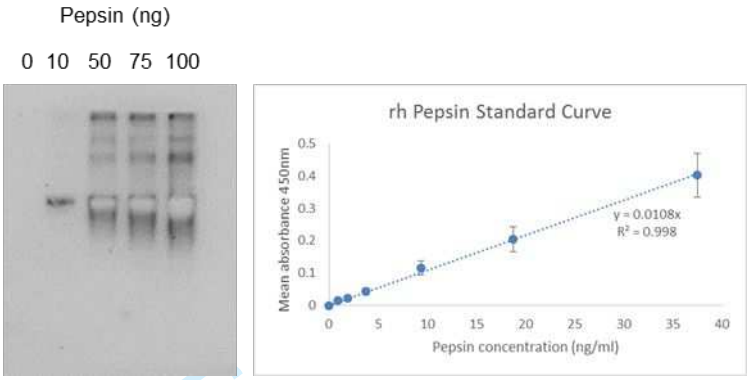
Studies of Salivary Pepsin in Patients with GERD

Caroline Race[1,5] Joanna Chowdry[2,5] Jean M. Russell[3,] Bernard M. Corfe[2,4,6] & Stuart A. Riley[1,4,6,7]

Section 1 – Pepsin ELISA Development

Determination of specificity of anti-pepsin monoclonal antibody.

Available monoclonal antibodies were surveyed for those with good user ratings, and proven application in both western immunoblot and ELISA. Santa Cruz sc-365680 was selected. The sensitivity of the antibody was determined in both immunoblot and ELISA.

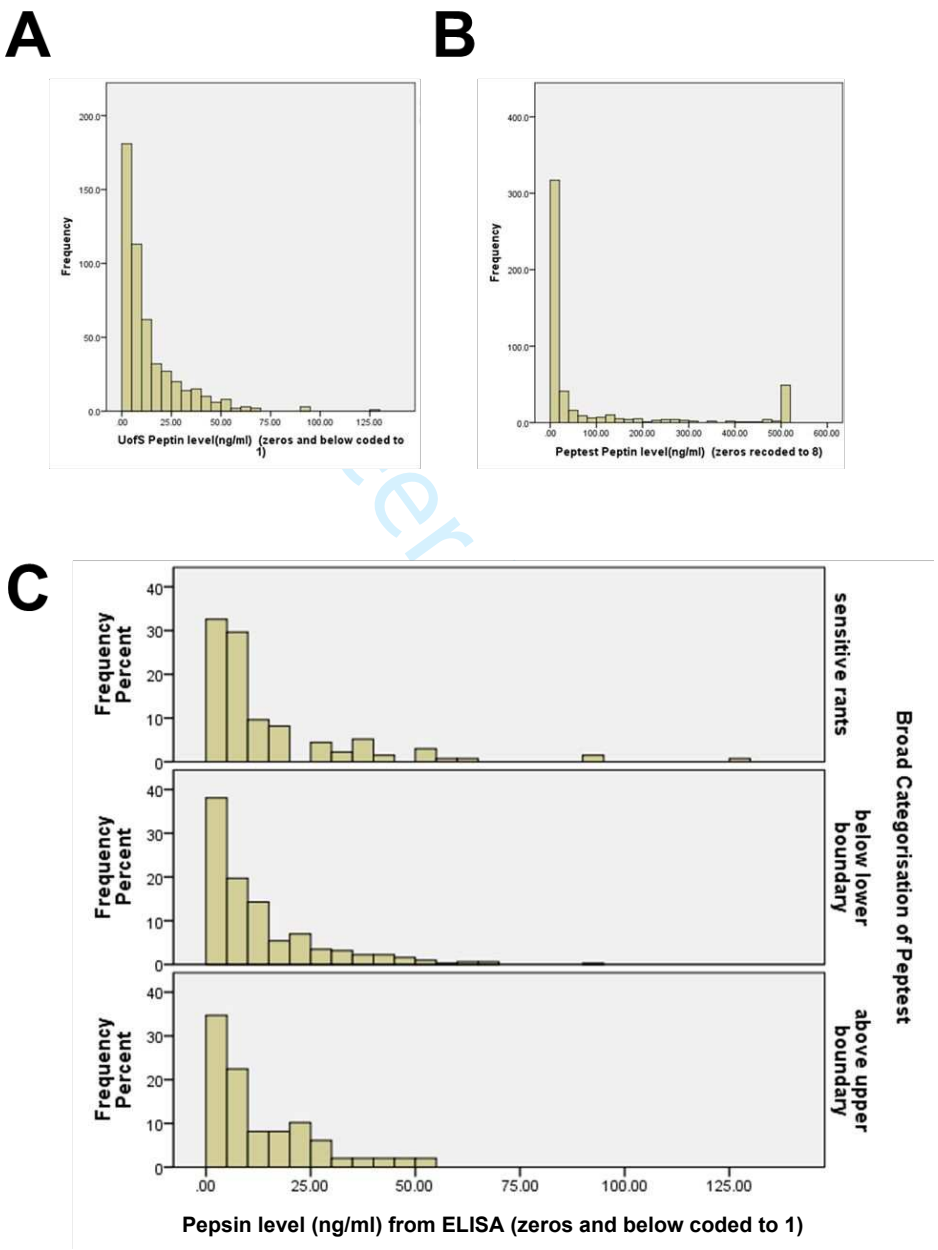


Left Panel For immunoblot assessment recombinant human pepsin A was loaded at 0-100ng into SDS-PAGE. The antibody cross-reacted with a single band (although breakdown products were detected at high antigen concentrations). The LoD for immunoblotting appeared to be <10ng.

Right Panel For ELISA, an indirect ELISA was developed and plates were coated with 0-40ng of recombinant pepsin, cross reactions were visualised with horseradish peroxidase conjugated anti-mouse secondary antibody. A linear relationship was observed in the 1-40ng range.

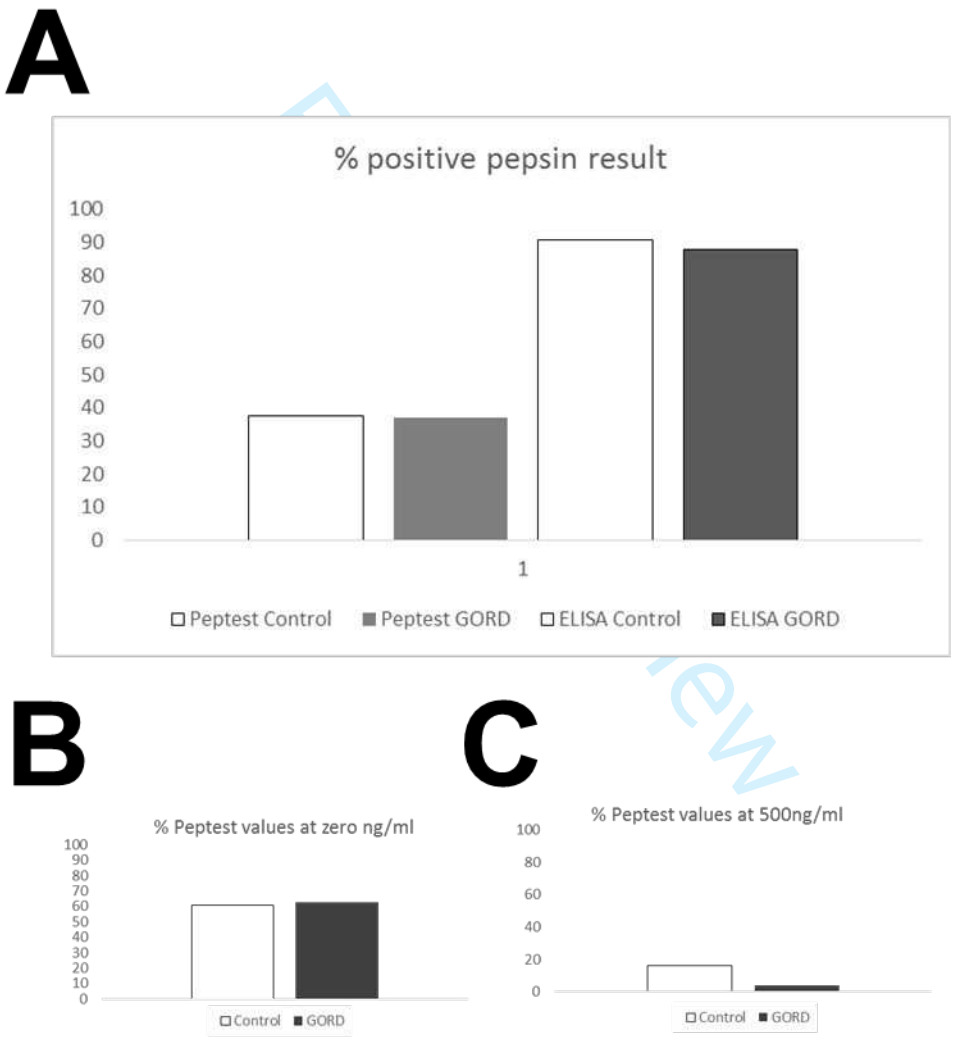
Section 2 – Comparison of Peptest and ELISA results

The distributions of data from 499 salivary analyses for pepsin ELISA and for Peptest are shown below. Peptest returned a large number of 0 scores which were converted to 8 (half of the stated limit of detection) in order to process the data. Pepsin ELISA follows a classical gamma distribution, whereas the Peptest data is hyperskewed with 73% of datapoints at either minimus or maximus. Panel C shows the distribution of pepsin quantifications according to three strata of Peptest scores (Upper boundary, lower boundary and sensitive range). There is no evidence of a significant difference in the distribution of pepsin ELISA data in these three categories (Kruskall Wallis=0.627. p=0.731).



Section 3 – Dichotomous analyses of Peptest and ELISA results according to RD Biomed's suggested thresholds

The data from Peptest and ELISA scores were dichotomised at 16ng/ml (RD Biomed's suggested diagnostic threshold) and were compared (Panel A). The rates of postives in the Control and GORD group are the same using this threshold by both methods. The Peptest data are hyperskwed: when only values of 0 were considered (Panel B), there was no difference in the negative score rate between GORD patients and controls. When only values att he upper limit of quantification were considered (Panel C) there are more positives in the control group.



Section 3 – Expression of pepsinogen in tongue

Expression analysis was undertaken for the expression profile of pepsin (the precursor peptide pepsinogen is shown). Analysis shown below indicates expression includes the tongue, suggesting potential for oral pepsin antigens. Expression Atlas accessed on 21st March 2018.