

RESEARCH ARTICLE

The Association of Pepsin and Reflux in US Individuals Self-diagnosing their Reflux like Symptoms.

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- (III) Provision of study materials: KHAB, JF, ADW
- (IV) Collection and assembly of data: KHAB, JF, ADW
- (V) Data analysis and interpretations: KHAB, ADW, PWD
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Abstract

Background: A high number of US citizens experience gastroesophageal reflux disease (GERD) symptoms at least once a month. These symptoms have a great impact on an individual's quality of life and present a huge burden on healthcare systems. Diagnosis of GERD like symptoms is not straight forward and many individuals do not have a diagnosis with current tests being invasive, expensive and often with low patient compliance. To speed the diagnosis process up there are many individuals seeking self-diagnosis and it became apparent that an easily accessible, rapid, non-invasive and cost-effective diagnostic test would be well received by individuals.

Methods: Seven hundred and ninety-three self-referral individuals experiencing reflux like symptoms provided up to three saliva samples. The first on waking, the other two samples provided either post-prandial or post-symptom. All saliva samples were sent to a central laboratory for pepsin analysis using a lateral flow device containing two unique human monoclonal antibodies (Peptest). Following analysis, the pepsin concentration in each saliva sample was determined using a PepCube reader and expressed in ng/ml.

Results: A total of 1834 saliva samples were analysed for pepsin. Sixty-two percent of individuals tested had one or more saliva sample pepsin positive and 38% of individuals had all samples pepsin negative. The highest pepsin concentrations were seen in post-prandial samples with the lowest pepsin concentrations significantly lower ($p=0.0127$) in the on waking samples. The US interstate pepsin concentration data is limited due to low numbers of self-diagnosing individuals tested to date. There was no difference in pepsin concentrations between genders.

Conclusion: The individuals self-diagnosing their reflux like symptoms were mostly those aged 40 to 70 years. The availability of a simple easy to use non-invasive test for self-diagnosis is now widely accessible across all states of the US.

Key Words: Salivary pepsin, Biomarker, Self-diagnosis, Reflux symptoms, Reflux diagnosis.

1. Introduction

Reflux is termed as the retrograde movement of gastric contents including the digestive enzyme pepsin into the esophagus and beyond, causing troublesome symptoms and mucosal damage.¹ Reflux can be an indication of diseases such as laryngopharyngeal reflux (LPR) and gastroesophageal reflux disease (GERD) with typical symptoms presenting as heartburn, regurgitation or atypical symptoms such as a chronic cough or mild hoarseness.² It was estimated that around 60 million Americans experience GERD like symptoms at least once a month.³ A study by De Bortoli *et al* 2018 reported approximately 40% of the US population complained of intermittent esophageal symptoms.¹ These symptoms have a great impact on a person's quality of life and add extra costs to healthcare providers.¹ Diagnostic methods for detecting reflux involve costly, time-consuming and invasive methods. These methods include endoscopy, 24-hour pH monitoring and more recently multichannel intraluminal impedance-pH (MII-pH) monitoring⁴ and have demonstrated to have poor sensitivity and reproducibility.⁵

Pepsin is a protease enzyme produced in the stomach from pepsinogen synthesized by gastric chief cells.⁶ Pepsin is present in gastric juice therefore, the presence of pepsin within the airways and salivary secretions makes pepsin an excellent biomarker for gastric reflux.⁷⁻⁹ A need for a less invasive, rapid and cost-effective diagnostic test which is easily accessible to clinicians and more importantly for individuals to access for self-diagnosing their reflux related symptoms led to the development of Peptest. Peptest uses lateral flow technology which contains two unique human pepsin monoclonal antibodies, one to detect pepsin and the other to capture pepsin.^{7, 10-12} A PepCube reader is used to measure and convert pepsin into ng/ml.

This study involved 793 self-referral individuals from across 45 states in the USA including Minnesota, Texas and Massachusetts all experiencing reflux related symptoms.

The aim of this study was to demonstrate how salivary pepsin can be used as a biomarker for reflux using Peptest as a non-invasive, fast diagnostic test for self-diagnosing reflux related symptoms.

2. Methods

2.1. Recruitment

This study analysed a total of 793 self-referral individual's saliva samples, all individuals were experiencing reflux related symptoms with no clinical diagnosis confirmed. These individuals were made up of 426 males and 367 females with a mean age of 52 years, ranging in age from 6 to 87 years. The number of individuals evaluated in the study was governed by those individuals who were self-diagnosing their reflux related symptoms.

2.2. Sample collection

All individuals were instructed to provide three saliva samples, the first 'on waking' prior to eating and cleaning their teeth maintaining an upright position, the other two samples were taken either post-prandial or post-symptom. The post-prandial samples were collected one hour after their main meal and post-symptom samples were collected within 15 minutes of experiencing reflux like symptoms. All individuals were advised to avoid using any medication to treat reflux 48 hours before providing their samples, for example antacids and alginate preparations but individuals could continue with their PPI treatment as PPIs do not prevent individuals from having reflux.¹³ All saliva samples were collected into 30 ml universal collection tubes containing 0.5 ml, 0.01M citric acid and stored at 4°C prior to the pepsin analysis within a maximum period of 7 days.

2.3. Sample analysis

The collection tubes containing the saliva samples were centrifuged at 4000 rpm for 5 minutes until a clear supernatant layer was visible. If no supernatant layer was 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]. The sample solution 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], which contains two unique human monoclonal antibodies; one to detect and one to capture pepsin in the saliva samples [Peptest, RD Biomed Limited, UK]. Fifteen minutes after introducing the sample for pepsin analysis into the well of the Peptest, the LFD was placed into the PepCube reader. The presence of pepsin in the saliva sample was determined by measuring the intensity of the pepsin Test line within the window of the lateral flow device. The minimum concentration of pepsin considered to be of relevance was ≥ 25 ng/ml and saliva samples containing this concentration and greater were considered as pepsin positive.



Figure 1: Schematic process for the collection and pepsin analysis of saliva samples by Peptest.

2.4. Statistical analysis

All the individual's data were anonymised prior to the completion of the study and the pepsin analysis. Unpaired 't' tests were completed between each sample collection time point and age group using the statistical package GraphPad Prism 8.2.0 [GraphPad Software, San Diego, CA 92018, USA]. P values <0.05 were considered statistically significant. The mean was displayed as \pm SEM.

2.5. 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 individuals seeking a self-referral. These were not patients recruited to take part in a clinical trial. Therefore, the ethical approval of this study was exempt. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from all the individual study participants. All participant data were anonymized prior to the final analysis of the data.

3. Results

All 793 self-referral US individuals provided saliva samples for pepsin analysis. This included 426 (54%) males and 367 (46%) females, with a mean age of 52 years. The age range of all individuals was 6-87 years. All individuals were instructed to provide three saliva samples, only 418 individuals adhered to this, a further 205 individuals provided two saliva samples and the remainder of individuals (170) provided one saliva sample. Altogether 1834 saliva samples were analysed using Peptest.

A total of 492 (62%) individuals had one or more saliva samples analysed as pepsin positive and 301 (38%) individuals had pepsin negative saliva samples. The mean pepsin concentration for all pepsin positive saliva samples was $188.38\text{ng/ml} \pm 4.9$. A total of 144 (29%) self-referring individuals had all three saliva samples pepsin positive at each collection point (Figure 2). The collection point with the highest pepsin concentration was seen in post-symptom at $250.7\text{ ng/ml} \pm 15.9$, with on waking having a lower pepsin mean concentration of $198.2\text{ ng/ml} \pm 13.5$. A statistical difference was observed between the two-collection points $P < 0.05$ ($p = 0.0127$).

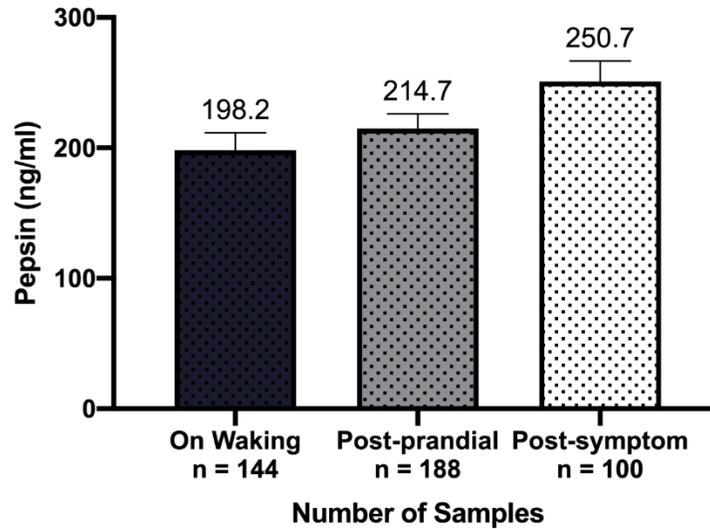


Figure 2: Mean pepsin concentration for self-referral individuals who produced three pepsin positive saliva samples at the different collection points. A significant difference ($p < 0.05$) was observed between post-symptom and on waking collection points. Mean displayed as SEM.

One hundred and seventy eight (36%) individuals had two saliva samples pepsin positive with the post-symptom collection point having the highest mean pepsin concentration of $204.7 \text{ ng/ml} \pm 15$, the lowest pepsin concentration was seen in the on waking sample at $165 \text{ ng/ml} \pm 15.3$. No statistical difference was observed. A further 170 (35%) individuals produced only one pepsin positive sample. Saliva samples collected post-prandial had the highest pepsin

concentration ($156.4 \text{ ng/ml} \pm 20.1$), the on waking sample had a significantly ($p < 0.05$) lower pepsin concentration at $99.0 \text{ ng/ml} \pm 14.70$. A significant difference was seen between the on waking sample and the post-symptom sample. A further breakdown of pepsin mean concentration was observed in the different states of America where six or more self-referral individuals produced a pepsin positive saliva sample (Figure 3).

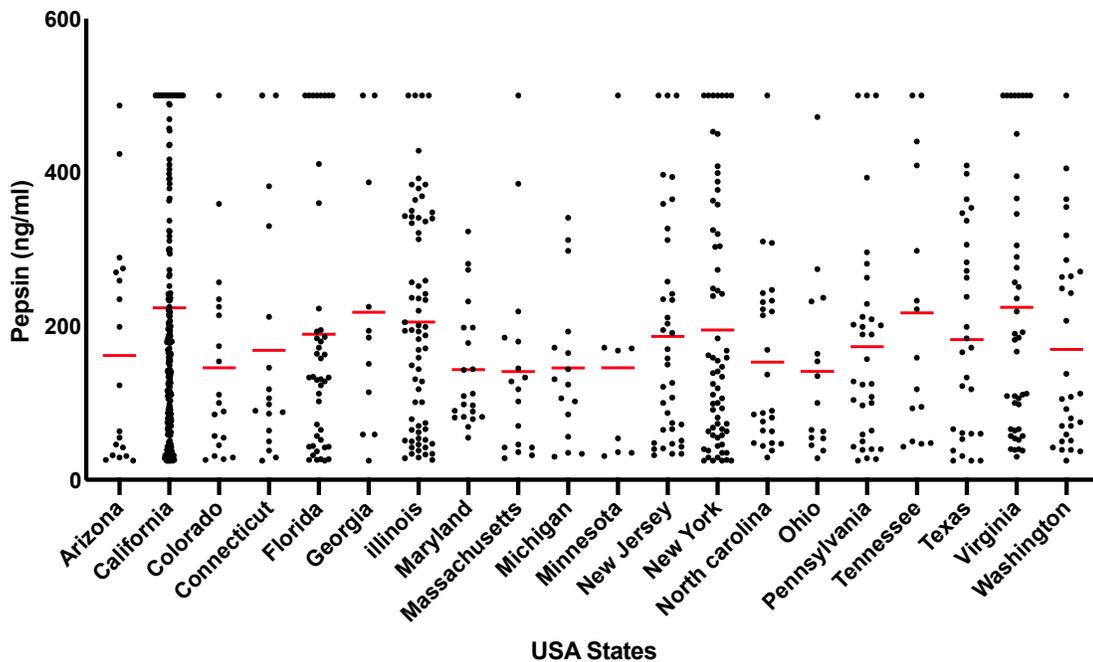


Figure 3: Mean pepsin concentration (indicated by the red line) for self-referral individuals across different states of America who produced pepsin positive saliva samples.

All 492 individuals who produced one or more pepsin positive saliva samples were compared by gender seen in Figure 4. The

female population had a higher mean pepsin concentration of $193.6 \text{ ng/ml} \pm 7.2$, the male population had a lower mean at $184.4 \text{ ng/ml} \pm 6.6$.

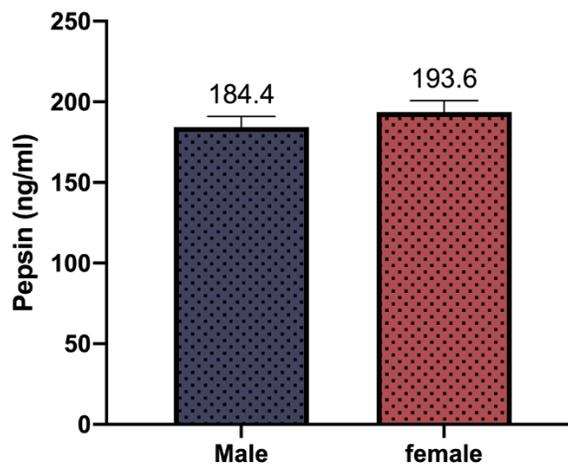


Figure 4: The mean pepsin concentration of USA male against female self-referring individuals who produced one or more pepsin positive saliva samples.

These individuals were split into groups categorised by age and sex, represented in Figure 5, the high n number for 41 – 70 year olds show this age range are seeking a reflux

diagnosis more than other age groups. The highest pepsin concentrations were seen in the age range of 0-30 years. No statistical difference was observed.

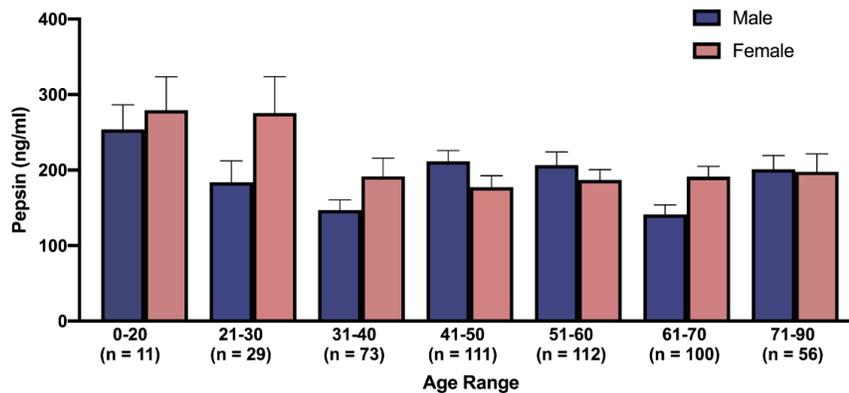


Figure 5: Age range and gender of self-referral individuals against a mean pepsin concentration in the USA (n = number of individuals).

4. Discussion

Gastroesophageal reflux disease (GERD) is the most prevalent gastrointestinal disease in the USA and a chronic disease which has been estimated to affect 60 to 70 million US individuals annually.¹⁴ GERD is very common and occurs across every age group, seeing year on year more individuals diagnosed with the disease. However, apart from those individuals who have received a diagnosis of GERD there is still a large and unknown number of individuals experiencing reflux like symptoms who have never received a diagnosis. To this day reflux symptoms remain the most common indication for GERD symptoms,¹⁵ individuals suffering with symptoms many for years, seek help, but diagnosis is time consuming and uses invasive and expensive diagnostics leading many individuals to self-diagnose their own medical condition.

The size of this group of individuals is unknown and many of them never seek professional help and regularly identify medical conditions themselves. These individuals use self-diagnostics on a very regular basis using various sources of information such as medical dictionaries, medical books, the internet, personal experiences as well as seeking advice from

friends and family with similar conditions and symptoms. We are told that self-diagnosis is potentially dangerous, there are pitfalls, risks of misdiagnosis and it is naturally discouraged by health professionals and physicians. However, self-diagnosis might be appropriate under certain circumstances. There are many popular over the counter (OTC) remedies for GERD and reflux sufferers, these range from antacids, histamine receptor antagonists (H2RAs) through to proton pump inhibitors (PPIs). Often OTC remedies are used on the assumption that the individual is capable of self-diagnosis and that the condition being treated is unlikely to cause any problems. However, these are not so simple remedies, and all have side effects especially if taken over a prolonged period and often without resolving the individual's symptoms. In the case of reflux this variable response is potentially due to excessive gastric acid not being a major factor in GERD and the substantial proportion of symptoms provoking reflux not being caused by acid reflux resulting in an incorrect diagnosis.¹⁶ The assumption that GERD was substantially an acid related disease led to the PPI test frequently being used to determine whether a patient's symptoms were acid related and if

the patient had GERD. However, the accuracy of this approach is now considered questionable.¹⁷

Other factors for the self-diagnosing individual which are important to consider are anxiety and depression.¹⁸ GERD is characterised by decreased quality of life not uncommon in a chronic condition and we know that anxiety and depression influence functional gastrointestinal disorders.¹⁹ Increased anxiety has been shown to increase the risk of GERD and in turn can enhance the perception of reflux symptoms.²⁰ The unanswered question is, is it more likely that the anxious and possibly depressed individual will seek self-diagnosis rather than seek help in a primary care or secondary care setting. Another interesting possibility of why individuals with reflux like symptoms seek a self-diagnosis for their symptoms are complications of failed anti-reflux surgery. It has been reported that anti-reflux surgery has declined largely due to concerns about short- and long-term complications²¹ for example insufficient symptom relief and the risk of developing new symptoms.²² It is also not uncommon for the GERD symptoms to return and this has been reported to happen in many patients.²³ It is therefore not so surprising that individuals would seek a further diagnosis (self-diagnosis) before considering further surgical intervention and treatment.

Gender is an important factor in GERD and in some countries, females are more likely to report with GERD than males and this can be by as much as a 40% difference.²⁴ We need to factor in that males are less likely to visit a physician and present for treatment or diagnosis. However, in the current study there were more males (54%) seeking a self-diagnosis than females (46%), a result which fitted with expectation. The total number of individuals who self-diagnosed from across 45 States in the US was 793 with a mean age of 52 years. Self-diagnosis can now go beyond using OTC medication with transient

relief of symptoms at best. The non-invasive and rapid reflux diagnostic Peptest has been available for US patients since 2013. Peptest is based on lateral flow technology and uses two unique human pepsin monoclonal antibodies for the detection of pepsin in salivary pepsin.^{7, 8, 25, 26} In the current study 1834 saliva samples were analysed for the presence of pepsin with 62% of the saliva samples found to be pepsin positive and the remaining 38% pepsin negative and therefore no evidence that their reflux like symptoms are due to reflux disease. The availability of Peptest has made self-diagnosis far easier saving individuals the time and expense of invasive reflux diagnostic tests (for example 24h pH monitoring, multichannel intraluminal impedance and pH monitoring, endoscopy). The other main advantage of using Peptest is speed of diagnosis taking only 20 minutes to diagnose the presence of the biomarker pepsin in an individual's saliva sample²⁷ and furthermore diagnosing the presence of reflux compared to invasive diagnostic tests which have a wait of at least 24 hours. There is also the question of compliance which is poor using invasive diagnostic tests.

Although the numbers of self-diagnosing individuals were low in the current study compared to the numbers of reflux sufferers in the US the data clearly showed the potential of using pepsin as a reflux diagnostic biomarker. With only limited data the two US states with individuals with the highest salivary pepsin concentrations were Virginia and California but as numbers of self-diagnosing individuals increases, we would expect to see similar pepsin concentrations in all individuals across all US states. The numbers of individuals using this method of reflux diagnosis will increase with the recent introduction of PepsinCheck by Peptest in May 2020 and which is now available across all 50 US States.

5. Conclusion

Following extensive clinical evaluation pepsin is now recognised as a biomarker for reflux disease and is gaining momentum as a non-invasive diagnostic test, for individuals and patients presenting with reflux like symptoms. It is clearly advantageous for them to receive a diagnosis before being prescribed medication and in the more serious cases, surgery. However, individuals in the US have busy lives, are apprehensive, non-compliant or simply do not have the finances to undertake invasive diagnosis tests. These individuals typically seek self-diagnosis for reassurance and speed of

diagnosis before undertaking treatment if required. With the introduction of an easy to use, non-invasive rapid diagnostic test in the US, self-diagnosis of reflux like symptoms will be that much easier.

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Conflicts of interest:

PWD is a director of RD Biomed Limited. ADW, JF and KHAB are employed by RD Biomed Limited.

Financial Disclosure: None

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