Pepsin as a Biomarker for Self-Diagnosing Reflux Associated Symptoms

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Abstract

Background: An increasing number of individuals are seeking self-diagnosis for their reflux related symptoms in the UK. Many individuals present with troublesome symptoms, such as regurgitation, heartburn and hoarseness and want a quick non-invasive diagnosis. Pepsin is produced in the stomach and is present in the backflow of gastric refluxate, therefore an excellent biomarker for reflux within a saliva sample. The aim of the study was to show how pepsin can be used as a biomarker for reflux, using the non-invasive diagnostic Peptest for the self-diagnosis of reflux related symptoms.

Methods: A total of 739 self-referral individuals [328 males, 411 females] provided three saliva samples for pepsin analysis. The first saliva sample taken 'on waking', the other two samples taken either post-prandial or post-symptom. The lateral flow device Peptest was used to detect pepsin in saliva and a PepCube reader used to determine the concentration of pepsin [ng/ml] in the sample.

Results: A total of 739 self-referral individuals provided saliva samples, the mean age of all individuals was 53 years. A total of 489 [66%] individuals had one or more saliva samples pepsin positive with an overall mean pepsin concentration of 205 ng/ml ± 48. 250 [34%] of individuals had pepsin negative saliva samples.

Conclusion: Peptest gives individuals access to a non-invasive diagnostic test for self-diagnosing reflux related symptoms, using pepsin as the biomarker for detecting salivary pepsin.

Keywords: Saliva Samples; Pepsin; Biomarker; Reflux Diagnosis; Reflux Symptoms

Abbreviations

GERD: Gastroesophageal Reflux Disease; LPR: Laryngopharyngeal Reflux; LFD: Lateral Flow Device

Introduction

A large number of individuals in the UK are seeking advice and help for reflux related symptoms presenting for example as heartburn, shortness of breath, regurgitation and hoarseness [1,2]. Many of these individuals are experiencing reflux like symptoms on a daily basis and want a quick diagnosis to identify and treat their health issues. Studies which have shown symptoms associated with reflux are common in the general population [3-5] and can be caused by a number of other health issues such as allergies, asthma, alcohol abuse and smoking, therefore making diagnosis difficult [6]. Procedures for diagnosing reflux include dual-probe 24 hour pH-monitoring, white light endoscopy and more recently multichannel intraluminal impedance/pHmetry [MII-pH] which are all invasive, have poor sensitivity and are time consuming and availability is limited [7-9].
Studies have shown reflux is associated with gastroesophageal reflux disease [GERD] [10] and laryngopharyngeal reflux [LPR] with characterized symptoms displaying as hoarseness, chronic cough, dysphonia and recurrent throat clearing [11] with a backflow of gastric stomach contents into the laryngopharynx [12]. Pepsin is present in gastric refluxate and exclusively produced in the stomach, the presence of pepsin within airway and salivary secretions confirms pepsin as a biomarker for gastric reflux [5,13,14]. Some studies have identified a correlation between the presence of pepsin in the laryngopharynx and reflux events and how pepsin causes damage to the oesophageal and laryngeal mucosae [15,16].

Using pepsin as a biomarker for reflux led to the development of Peptest, which is a non-invasive, rapid diagnostic procedure used to identify pepsin within a saliva sample [5]. Peptest uses lateral flow technology containing two unique human pepsin monoclonal antibodies, one used to detect pepsin and the other to capture pepsin [17-19]. A PepCube reader is used to measure and determine pepsin concentration in ng/ml.

**Aim of the Study**

The aim of this study was to demonstrate how pepsin can be used as a biomarker for reflux by using Peptest as a first line non-invasive diagnostic test for self-diagnosis of reflux related symptoms.

**Methods**

**Recruitment**

A total of seven hundred and thirty-nine self-referral individuals’ samples were analysed in this study, all experiencing reflux related symptoms with no clinical diagnosis confirmed. These individuals were made up of 328 males and 411 females with a mean age of 53 years, ages ranged from one month to 90 years.

**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, then the other two samples 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 any medication to treat reflux 48 hours before providing their samples.

All saliva samples were collected into 30 ml collection tubes containing 0.5 ml 0.01M citric acid and stored at 4°C prior to pepsin analysis.

**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) containing two unique human monoclonal antibodies; one to detect and the other to capture pepsin in the saliva samples [Peptest, RD Biomed Limited, UK].

![Figure 1: Schematic process for the collection and analysis of saliva samples for the use of Peptest.](image-url)
Fifteen minutes after introducing the 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. Pepsin concentrations ≥25 ng/ml were considered positive.

**Statistical analysis**

All individual's data were anonymised prior to the completion of this study and the analysis performed. 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 ± SEM.

**Results**

A total of 739 UK self-referral individuals provided saliva samples for Peptest analysis. This total was made up of 328 males and 411 females, the female/male ratio was 1.25:1. The mean age for all individuals was 53 years (range 1 month to 90 years). All 739 self-referral individuals were instructed to provide three saliva samples. Three hundred and fifty-seven individuals provided three saliva samples, 219 individuals provided two saliva samples and the remaining 163 individuals provided just one saliva sample. A total of 1672 saliva samples were analysed.

Saliva samples were pepsin negative in 250 (34%) of the individuals (112 males and 138 females). The remaining 489 individuals had one or more saliva samples pepsin positive [66%]. The mean pepsin concentration for all positive pepsin samples was 205 ng/ml ± 4.8. All three saliva samples at each collection point were pepsin positive in 152 of the individuals (Figure 2). The highest of pepsin concentration was seen at the post-symptom collection point 253.7 ng/ml ± 14.5. The ‘on waking’ sample had a slightly lower mean concentration of 241.8 ng/ml ± 13.5. No significant difference was observed at the different collection points.

![Figure 2: The pepsin concentrations from each collection point taken from individuals with all three samples pepsin positive.](image)

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A total of 185 self-referral individuals had two samples pepsin positive. The highest pepsin concentration was in the post-symptom sample at 197.5 ng/ml ± 13.6. The lowest was in the ‘on waking’ sample [156.3 ng/ml ± 15.0]. There was no significant difference between each collection point. One hundred and fifty-two individuals [31%] had only one sample pepsin positive. The highest pepsin concentration was seen in the post-symptom saliva sample at 163.2 ng/ml ± 18.4 and the lowest pepsin concentration was 108.5 ng/ml ± 16.9 in the ‘on waking’ saliva sample. A statistical difference [P < 0.05] was observed between ‘on waking’ and post-symptom samples.

Figure 3 represents the mean ±SEM pepsin concentration of self-referral individuals who had three, two or one saliva sample pepsin positive. The mean pepsin concentration of individuals with all three saliva samples pepsin positive was 246.9 ng/ml ± 7.8. The mean pepsin concentration for individuals who had two pepsin positive saliva samples was 181.4 ng/ml ± 7.1 and individuals who produced just one pepsin positive sample had a mean of 136.3 ng/ml ± 8.9.

Figure 4 compares the pepsin concentration in both males and females from the 489 self-referral individuals who had pepsin positive samples. It was observed the mean [SEM ±] pepsin concentration was higher in females than males, with no significant difference.
The age and sex of self-referral individuals who supplied a pepsin positive saliva sample are illustrated in figure 5; no significant difference was observed. The females generally had a higher salivary pepsin concentration at all ages except in the age range of 51 - 60 years. The demographic information for all the self-referral individuals who produced a pepsin positive saliva sample is represented in table 1.

**Figure 5:** Pepsin concentrations of male and female individuals across the study age range.

<table>
<thead>
<tr>
<th>Age Range</th>
<th>Male [n =]</th>
<th>Female [n =]</th>
<th>Total Individuals [n =]</th>
</tr>
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<tr>
<td>0 - 20</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>21 - 30</td>
<td>13</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>31 - 40</td>
<td>23</td>
<td>30</td>
<td>53</td>
</tr>
<tr>
<td>41 - 50</td>
<td>57</td>
<td>51</td>
<td>108</td>
</tr>
<tr>
<td>51 - 60</td>
<td>37</td>
<td>74</td>
<td>111</td>
</tr>
<tr>
<td>61 - 70</td>
<td>45</td>
<td>72</td>
<td>117</td>
</tr>
<tr>
<td>71-90</td>
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<td>69</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>274</td>
<td>489</td>
</tr>
</tbody>
</table>

**Table 1:** Demographic information of 489 self-referral individuals who provided a pepsin positive saliva sample.

**Discussion**

It has been well documented that routine diagnostic procedures for the diagnosis of reflux are highly invasive and time-consuming, resulting in high costs for healthcare providers [20]. The current diagnostic procedures for reflux related diseases include endoscopy, pH monitoring and multichannel intraluminal impedance/pH monitoring [MII-pH]. These are uncomfortable procedures for patients to experience and in some cases even require an overnight stay which increase the overall cost of the diagnostic procedure [21].

The main aim of this study was to use Peptest as an alternative and non-evasive diagnostic test, at a low cost and rapid way of diagnosing reflux using an individual’s saliva sample. The use of a simple saliva sample gives individuals an easy collection procedure to follow from the comfort of their own home. The Peptest uses lateral flow technology to detect the presence of pepsin within a saliva sample, which is converted into ng/ml pepsin. Past studies have validated the use of Peptest, which was shown to have 85% sensitivity and speci-

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ficity ranging from 60% to 100% [5,22]. Previous studies have reported using pepsin as a biomarker to confirm the presence of reflux in saliva with positive and reliable outcomes, even amongst individuals who experience non-acidic reflux [23,24]. Pepsin is a digestive enzyme primarily found in the stomach, therefore its presence within saliva is a good indicator of reflux [25]. Pepsin plays a role in diseases such as gastroesophageal reflux disease [GERD] [3,5,10] and laryngopharyngeal reflux [LPR] [22,26]. Both GERD and LPR refer to a backflow of gastric contents but differ in symptoms indicative of the diagnosis. The large size of pepsin makes it easily detected in saliva after gastric reflux [27]. A recent study by Haddad., et al. investigated the presence of salivary pepsin A in gastroesophageal reflux episodes in children undergoing impedance probe monitoring and found pepsin A present in the saliva of children being investigated for GERD, it was reported pepsin A was associated with a reflux episode [28]. Another study by Bor., et al. aimed to validate pepsin detection in saliva using ten classical GERD patients and ten LPR patients all undergoing impedance testing. The study used Peptest to detect salivary pepsin and concluded Peptest as a good first-line diagnostic test to confirm reflux [29]. Several studies have demonstrated pepsin to be a good biomarker for GERD and LPR in patients [24,30-33].

GERD is increasing in prevalence worldwide and becoming a burden to the global healthcare systems, it’s prevalence in the USA is estimated to be 27.8% and in Europe to be 25.9% [34]. The increase is partly due to the lifestyle choices of individuals along with diet and alcohol consumption which all have a role in the increase of reflux associated symptoms [35,36].

The 739 individuals in this study were based in the UK and had no previous diagnosis of reflux with an age range between one month to ninety years. All individuals were experiencing troublesome reflux like symptoms and used Peptest to self-diagnose their symptoms. This study used Peptest to analyse 1672 saliva samples, from individuals who were seeking to confirm reflux and plan a course of treatment to better their quality of life. The highest number of individuals seeking help were in the age range 41 - 70 years, indicating middle aged people are more affected by reflux like symptoms and more likely to seek help in identifying if their symptoms are reflux related. A total of 250 individuals had saliva samples pepsin negative and 489 individuals had one or more saliva samples pepsin positive [> 25 ng/ml]. A multicentre study from 2019 looked at five UK voice clinics reviewing a total of 1011 LPR patients who had previously received unclear and contradictory results for reflux but presented with symptoms of LPR. Peptest was used as the diagnostic method, with an overall sensitivity and specificity of between 85% to 100% [5,22], the overall mean pepsin concentration seen in the patient groups was 131 ng/ml [22]. Interestingly, the overall pepsin concentration was higher in this self-referral study at 205 ng/ml.

This study shows how reflux related symptoms have a big impact on an individual’s quality of life, so much so that it promotes a need to source a self-diagnosis. The results often led to the individual seeking medical advice on how to further manage their symptoms. It becomes another route for individuals who have not been clinically diagnosed or in some cases misdiagnosed by clinical practitioners. The rapid testing that Peptest offers guarantees no waiting time, making it much more of a convenient test for individuals to fit into a busy lifestyle. Peptest can also be used as a rapid diagnosis within a clinical setting at a lower cost.

This study has demonstrated and validated the use of Peptest as a first line non-invasive diagnostic test. One limitation of the study was the higher age range and the fact that it was self-referral may reflect on the low number of younger individuals in this study.

Conclusion

Knowing that many individuals throughout the UK suffer with reflux related symptoms, makes it important for individuals to have access to alternative non-invasive diagnostic procedures. Peptest uses pepsin as a biomarker to give a self-referral diagnostic test which is validated, rapid and easy to use for confirming the presence of reflux disease.

Conflict of Interest

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

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