Nasal and salivary pepsin as a biomarker for gastro-esophageal reflux in chronic rhinosinusitis*

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Abstract

Background: Gastro-esophageal reflux (GER) may be a contributing factor for some patients with chronic rhinosinusitis (CRS). The aim of the present study was to investigate if Peptest, an immunoassay for pepsin detection, could be used as a biomarker for GER in CRS.

Methodology: Peptest was used to analyse 3 saliva and 3 nasal samples for pepsin A in 62 CRS-patients and 62 age and gender matched healthy controls. The results were correlated to 24-hour impedance pH-monitoring and symptom questionnaires.

Results: Patients with CRS did not have more abnormal Peptest measures compared to healthy controls, 39 patients and 48 controls, respectively. The presence of abnormal Peptests did not correlate to proximal reflux in CRS-patients. Patients with high GerdQ scores did not have more positive Peptests than those without.

Conclusions: These results question the value of Peptest as screening tool for GER in CRS.

Key words: Chronic rhinosinusitis, gastro-esophageal reflux, pepsin, Peptest

Introduction

Chronic rhinosinusitis (CRS) is a common chronic condition affecting up to 15 % of the adult population [1]. It influences quality of life and working capacity by the inflammation of the mucosa of the nose and paranasal sinuses. The aetiology of the inflammatory process is not completely understood, but a common hypothesis is that inappropriate immune responses to foreign agents results in CRS. Stimulation of T cells by *Staphylococcus aureus* protected in biofilms is an example of such immune response. Whether severe cases of non-allergic rhinitis, allergic rhinitis, occupational rhinitis and CRS not responding to conventional treatment, could share a common underlying pathophysiology, is unclear [2].

The lack of etiological clues impairs the development of evidence based therapeutic strategies. The high number of patients with CRS failing to respond to medical and surgical treatment represents a major challenge both for the individual patients as such, but also for general practitioners as well as for specialists, and for society in general.

The role of Gastro-esophageal Reflux (GER) in upper airway disorders including CRS has been debated for decades [3-5]. GER has an established role in the pathogenesis of dental erosions [6], asthma [7], chronic cough [8], laryngitis [9] and possibly also in chronic otitis media [10]. Its role in CRS is unclear [3, 11, 12]. In the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 (EPOS 2012) it is therefore stated that more research is needed to enlighten the role of GER in CRS [13].

In a recent study, we demonstrated that patients with gastro

esophageal reflux disease (GERD) had high scores on SNOT-20, a questionnaire for sino-nasal quality of life [14]. In another study, we found that abnormal gastroesophageal reflux was significantly more prevalent in CRS-patients than in healthy subjects as evaluated by 24 -hour esophageal impedance pH monitoring, the gold standard for the investigation of GER [15]. Twenty-four-hour impedance pH-monitoring is a resource-demanding procedure, and not easily available. Alternative diagnostic methods are pH- monitoring only or endoscopic examinations. The former will not detect episodes of non-acid reflux, and the latter will only detect those with visible pathology in the esophagus. Thus, it is warranted to find alternative and still valid methods for investigation of GER in CRS. Reflux of gastric contents through the esophagus into the airways includes acid, pepsin, mucus, bile acids, pancreatic enzymes, and remnants of food and drinks. Pepsin has been used as a diagnostic biomarker of GERD. A study of paediatric patients demonstrated an association between pepsin in saliva and findings at 24- hour impedance pH- monitoring [16]. Also, pepsin concentrations in nasal lavage fluid were high, and correlated well with esophageal acid exposure [17]. However, these results were not reproduced in another study which concluded that detection of salivary pepsin was an imprecise method at least for investigation of reflux in children with CRS [18].

The aim of the present study was to evaluate if Peptest, a widely used immunoassay method for pepsin detection, could be used as a biomarker of GER in CRS. Furthermore, we wanted to evaluate if pepsin could be measured in nasal secretions and if so, compare the results with measures from healthy controls.

Methodology

Patients and healthy controls

This was a prospective controlled age and gender matched study, performed on an outpatient basis. Consecutive patients above 18 years of age, referred to the ENT department, Stavanger University Hospital and diagnosed with CRS with or without polyps according to the EPOS 2012 criteria [13] were invited to participate. Sixty two CRS patients were included, 33 men and 29 women. A similar number of age and gender matched healthy controls were recruited at different jobs, among patient's friends, neighbours, colleagues and among the investigator`s friends, family, colleagues and neighbours (Table 1). Median age in patients and healthy controls was 46 and 46.5 respectively (p=0.81). The same investigator handled all patients and healthy controls.

Exclusion criteria were use of anti-reflux medication on a daily basis. Symptoms of reflux were not an exclusion criteria per se. Nasal corticosteroids were not allowed the day of investigation.

Peptest

Peptest® (RD Biomed Ltd, Cottingham, UK) is an enzyme linked

Table 1. Subject characteristics.

	CRS-patients	Healthy controls
Age, years	46	46.5
Gender, men/ women	33/ 29	33/ 29
Smoking	10	1
BMI, median	25.1	25.9
Self-reported asthma	18	2
Ongoing treatment of reflux	0	0
Self-reported allergy	16	8

CRS= Chronic rhinosinusitis, BMI= Body Mass Index

immunoassay for detection of pepsin in saliva. This non-invasive test is based on a lateral flow device technology. It consists of antibodies to human pepsin and is specific to pepsin A, the isoform secreted only in the stomach [19, 20]. A control band on the device indicates a correctly performed test and a second line indicates presence of pepsin, in the sample, defining a positive or negative test. The intensity of this line is proportional to the quantity of pepsin, and by using a reader, LFDR101, the concentration of pepsin in ng/mL is obtained [20]. Both the sensitivity and specificity of Peptest has been claimed to be 87 % [21], but recent meta-analysis suggests the sensitivity and the specificity to be lower, 64% and 65% respectively [22]. The lower limit for detection of pepsin with Peptest is 16ng/ mL given by the manufacturer [20]. To the best of our knowledge, Peptest has not previously been used on nasal secretions.

CRS patients and healthy controls delivered 3 tubes with saliva and 3 tubes with nasal secretion (roughly 1 mL in each) for pepsin analysis. We strictly followed the recommendations from the manufacturers; "Sample one should be taken in an upright position within 15 minutes of waking up from overnight sleep before eating or brushing teeth, the second sample should be taken one hour following the main meal of the day and the third sample should be taken one hour following the next main meal of the day" [23]. Subjects without rhinorrhoea used 0,5 mL sterile water for irrigation of each nostril to be able to deliver samples. The tubes for investigation contained 0,5 mL of 0,01 M citric acid for conservation, and the participants stored the samples in a refrigerator until analysis within 7 days, as recommended by the manufacturer [21]. Each sample was centrifuged at 5000 rpm for 5 minutes, 80 µl of the supernatant was extracted, and 240 µL migration buffer was added. This was vortex-mixed for 10 seconds before 80 µL was transferred to the pit of the Peptest device. The test was read as positive or negative after 15 minutes and quantification done by using the LFDR reader.

The same investigator performed all the tests on both CRS patients and healthy control subjects.

Table 2. Symptom scores in patients with chronic rhinosinusitis and healthy controls.

	CRS-patients	Healthy controls	p*
GerdQ, median (IQR)	6 (5.3-7.0)	6 (5.5-6.0)	0.25*
Number participants with GerdQ ≥ 8	12	3	0.04**
VAS-CRS, median (IQR)	50.5 (31.7- 65.8)	15.5 (8.8- 20.1)	< 0.001*
SNOT-20, median (IQR)	34.5 (20.3- 52.5)	4 (2.0- 10.0)	< 0.001*

Questionnaires were available for 60 matched pairs of patients and controls. *Wilcoxon test for paired samples, **McNemar test for paired samples, CRS= Chronic rhinosinusitis, IQR= Interquartile Range, GerdQ= Gastroesophageal Reflux Disease Questionnaire, VAS-CRS = Visual Analogue Scale for chronic rhinosinusitis, SNOT-20= 20-item Sinonasal Outcome Test.

24-hour combined pH-impedance monitoring

Forty-six patients completed 24-h combined pH-impedance monitoring, with recordings for 18 - 26 hours. The impedance pH-catheter (ZAN-BG-44, Sandhill Scientific, Inc; Highland Ranch, CO, USA) was positioned trans-nasally into the oesophagus, with the proximal pH-electrode located 5 cm above the lower esophageal sphincter and the impedance electrodes 2, 4, 6, 8, 10, 14, 16 and 18 cm above the lower esophageal sphincter [15].

Questionnaires

Validated questionnaires were used to evaluate the clinical symptoms of GERD and CRS in patients and controls: Visual Analogue Scale (VAS-CRS) is a psychometric response scale used to measure subjective sino-nasal symptoms [13]. The 20-item Sinonasal Outcome Test (SNOT-20) is a validated self-administered quality of life instrument specific for patients with symptoms of rhinosinusitis [24]. The Gastro-Esophageal Reflux Disease Questionnaire (GerdQ) was used to record classical symptoms of GERD. It is a validated six item, self- administered questionnaire, scoring symptoms of reflux and dyspepsia separately, with reflux symptoms increasing the score and dyspepsia decreasing it. Absence of any such symptoms gives a total score of 6 and scores above 8 indicate reflux disease [25]. The Peptest scores were compared to GerdQ and VAS-CRS and SNOT-20. GerdQ, VAS-CRS and SNOT-20 in CRS-patients and healthy controls were also compared.

Statistical analysis

As data were not normally distributed, non-parametric statistics was used. Summary statistics are reported as median and interquartile range (IQR) for continuous variables and numbers and percentages for categorical variables. Differences between
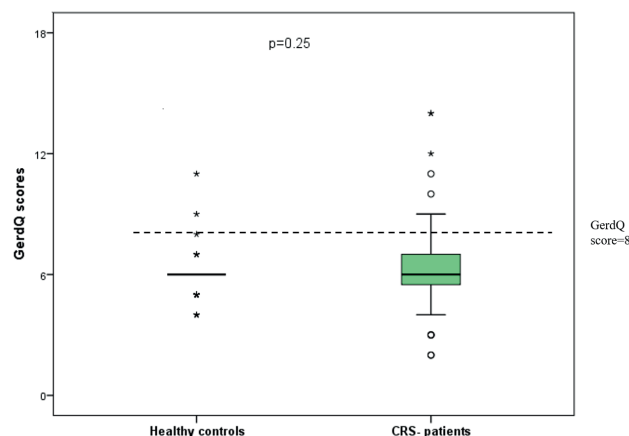

Figure 1. GerdQ scores in healthy controls and CRS-patients. No significant difference in GerdQ, Gastroesophageal Reflux Disease Questionnaire, scores between CRS-patients and healthy controls, Wilcoxon, p=0.25. GerdQ is a validated six item, self- administered questionnaire, scoring symptoms of reflux and dyspepsia separately, with reflux symptoms increasing the score and dyspepsia decreasing it. Absence of any such symptoms gives a total score of six and scores above eight indicate reflux disease. Three healthy controls and 12 patients had a GerdQ score ≥ 8, which is the level indicating GERD (p=0.08). CRS= Chronic rhinosinusitis.

patients and controls were examined using the paired samples Wilcoxon test for continuous data and McNemar test for categorical data. Correlations between variables where calculated using Spearman`s Rank Correlation (rho).

The statistical analyses were done using SPSS ver. 24.0 (Statistical Package for Social Science, Chicago, IL, USA).

Ethics and approval

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by Regional Norwegian Committee for Medical and Health Research Ethics approved the study (REK Vest 2010/2030). All participants were included after written informed consent.

Results

Sixty-two CRS patients were included, 33 men and 29 women, and 62 controls (Table 1). There was no significant difference in Body Mass Index (BMI) between the CRS-patients and healthy subjects, median 25.1 and 25.9 respectively (p=0.46) (Table 1). Questionnaires were available for 60 matched pairs of patients and controls. Total GerdQ scores were not significantly higher in the CRS-patients compared to the healthy controls (p=0.25) but 12 patients had GerdQ score ≥ 8, which is the level indicating GERD, compared to 3 in the control group (p=0.04) (Table 2). The patients had significantly higher SNOT-20 and VAS-CRS scores, compared to the controls (Table 2).
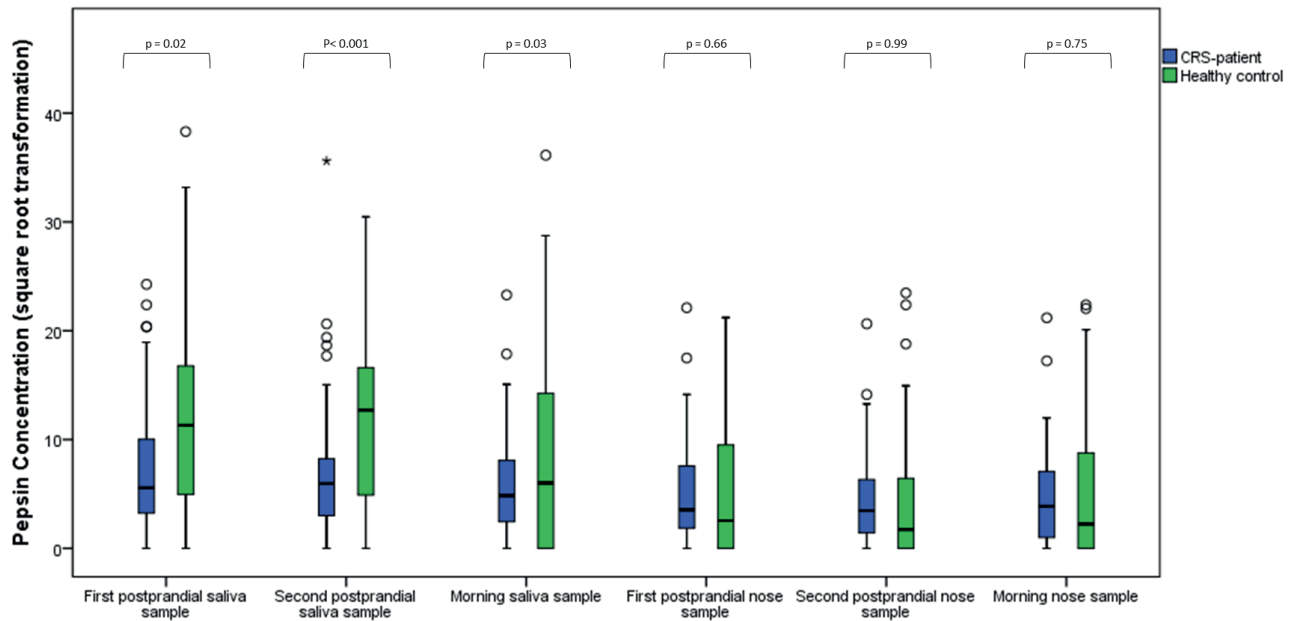There was no significant difference in number of positive

Figure 2. Pepsin concentration in nasal secretion and saliva in healthy controls and CRS-patients. Pepsin level in nasal secretion and saliva measured one hour after two different meals and within fifteen minutes after waking up in the morning. There were no significant differences in pepsin concentration in nasal samples between CRS-patients and healthy controls, but significantly higher pepsin concentration in saliva in healthy control subjects compared to CRS-patients, paired sample Wilcoxon test. The concentrations are square root transformed for improved readability.
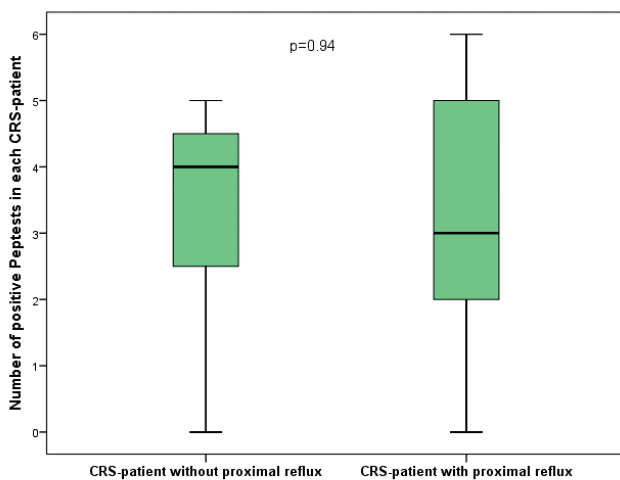

Figure 3. Total number of Peptest positive samples per CRS-patient with and without proximal reflux, diagnosed with 24-hour pH-impedance monitoring. There was no significant difference, p= 0.94.

salivary Peptests in patients and healthy controls (Table 3). Abnormal Peptest results in saliva samples, when defined as two or more positive Peptests or Peptest levels in one test ≥ 100 ng was found in 39 patients and 48 controls (p= 0.08). The concentration of pepsin was significantly higher in the saliva of healthy controls compared to CRS-patients (Table 3, Figure 2). There were large variations in pepsin levels both in patients and controls, particularly in the latter.

There was significantly higher number of positive Peptests in

the second postprandial nasal sample in patients compared to healthy controls (p=0.02), but not in the other samples (Table 3), and the levels of pepsin in nasal samples did not differ between the two groups (Table 3, Figure 2).

There was no significant difference in number of positive Peptests between patients with abnormal proximal reflux compared to those without (p=0.94) (Figure 3), and no correlation between the number of positive Peptests and number of proximal reflux (Spearman rho 0.13, p=0.42), nor between the number of positive Peptests and number of distal acid reflux episodes (Spearman rho 0.06, p=0.71). CRS patients with high GerdQ scores did not show higher concentration of pepsin in saliva or nasal secretion and number of positive tests was not significantly different between the two groups (p= 0.71). There was no correlation between GerdQ scores and number of positive Peptest in the healthy controls (Spearman rho 0.17, p=0.18) or patients (Spearman rho 0.15, p=0.24).

Discussion

In this study we have demonstrated that patients with CRS did not have more pepsin A in saliva or nasal secretions as measured by Peptest compared to healthy controls. CRS patients with abnormal proximal reflux did not have more samples positive for Peptest than patients without. As a group, there were more patients with high GerdQ scores for classical GERD symptoms compared to the healthy controls. However, less than 40% of patients with EER may have the classical symptoms of reflux,

Table 3. Pepsin concentration in saliva and nasal secretions, as measured with Peptest.

	Number of positive Peptests (%)			Pepsin concentration, ng/ mL, median (IQR)		
	CRS-patients	Healthy controls	P*	CRS-patients	Healthy controls	P**
First postprandial saliva sample	35 (67.3)	34 (65.4)	1.00	31.0 (10.0-101.0)	128.0 (24.3-282.0)	0.02
Second postprandial saliva sample	34 (60.7)	37 (66.1)	0.69	35.5 (8.5-68.3)	161.5 (21.5-278.5)	<0.001
Morning saliva sample	30 (51.7)	27 (46.6)	0.71	23.5 (6,0-68.3)	36.0 (0.0- 238.5)	0.03
First postprandial nose sample	27 (47.4)	17 (29.8%)	0.11	12.5 (3.3-58.8)	6.5 (0.0-93.3)	0.66
Second postprandial nose sample	26 (45.6)	13 (22.8)	0.02	12.0 (1.0- 42.1)	3.0 (0.0- 44.0)	0.99
Morning nose sample	26 (42.6)	17 (27.9)	0.15	15.0 (1,0-52.5)	5,0 (0,0-80.0)	0.75

Peptest results in saliva and nose samples collected at three different times during a day in CRS patients and healthy controls. There were significantly more positive Peptests in CRS-patient`s second nasal postprandial sample but no significant difference between the other groups regarding number of positive tests. There was significantly higher pepsin concentration in saliva samples from healthy controls, but no significant difference in pepsin concentrations in nasal samples between CRS-patients and healthy controls regarding pepsin concentration. The number of paired samples available varies from 48 to 61. CRS= Chronic rhinosinusitis, IQR= Interquartile range, *Categorical data tested with McNemar test, ** Continuous data tested with Wilcoxon test.

meaning that the questionnaire does not have an important role in the evaluation of these conditions. Patients with high GerdQ scores or verified proximal reflux did not have more positive Peptests than the controls. These findings question the validity of the Peptest in the evaluation of GER in CRS.

Pepsin is a proteolytic enzyme responsible for digestion of proteins in the stomach. As pepsin A originates from pepsinogen secreted from gastric chief cells in the stomach, the finding of pepsin A in the esophagus, mouth or respiratory tract should be a diagnostic marker of reflux [26]. The nasal mucosa has limited protective capacity against the refluxate, and could be more sensitive to its injurious influence [12]. Pepsin is most harmful in its acidic state. However, it can cause injury with a pH up to 6,5 and is not irreversibly denatured until pH reaches 8. This implies that when pH in refluxate is above 4, and thus not detected by pH monitoring, there can still be active pepsin in the refluxate. Besides, theoretically, inactive pepsin can be reactivated and become harmful by a decrease in pH due to acid drinking or later acidic reflux events [27].

The high number of positive Peptests in both patients and healthy controls in this study supports the concept of physiological, mainly postprandial reflux. Every healthy individual refluxes small amounts of gastric contents, mainly after meals, most of them without having symptoms, and without having GERD [11]. Less is known about reflux to the airways in healthy subjects. The high number of positive Peptests both in those with and without CRS may indicate individual differences in sensitivity to refluxate including pepsin, i.e. a certain level of pepsin may create disease in some individuals, but not in others. This theory has been supported by pH- impedance monitoring

of the esophagus combined with symptom registration, with an estimation of Symptom Association Probability (SAP). SAP gives an indication of a time correlation between symptoms and reflux episodes, and it has been shown that exposure to refluxate that normally is considered physiological, may give symptoms in some patients [28]. Thus, it may be speculated whether the same differences in vulnerability also exists in the nose, whether a certain level of pepsin may contribute to the development of CRS in certain predisposed individuals, but not in others. The lower concentration of pepsin in patients compared to controls may partly be explained by gastroesophageal reflux inducing hyper salivation [29]. The phenomenon has also been described in healthy persons when stimulating the esophagus with hydrochloric acid [30]. Hyper-salivation may dilute the concentration of pepsin in patients and thus act as a confounder in this study.

Peptest is supposed to give snapshot-information about the presence or absence of pepsin in saliva and nasal secretions. The concentration of pepsin in saliva varies during the day and decreases rapidly after an episode of reflux [31]. Thus, saliva samples should be obtained soon after reflux events to detect the pepsin [16]. Inappropriate timing can reduce real-life sensitivity of the Peptest though it has high technical sensitivity [32]. Reflux appears mostly after meals, and corresponding symptoms of GERD is significantly highest 1 to 2 hours post-prandially [31]. In the present study, we tested for pepsin 3 times a day, determined by time of waking up and time of meals and not by symptomatic reflux episodes. Hence, there could be episodes of reflux not detected with Peptest both in CRS-patients and controls, most likely more frequently in the former. That this is a valid assumption is supported by the fact that pH-impedance of the esopha-

gus, monitoring gastro-esophageal reflux continuously over a prolonged period of time, demonstrated both an increased incidence and severity of reflux in CRS-patients [15]. Then it may be discussed to what extent detection of refluxate of gastric content, including pepsin, in the esophagus implies its presence and pato-physiologic role even in the upper airways. For the time being, there is no conclusive answer to that question. Two different pathophysiological mechanisms have been proposed to explain how GERD may contribute to airway manifestations, through a vagal reflex or a direct toxic effect. As to CRS, the theory is that a neuronally induced nasal mucosal oedema gives ostial obstruction [33]. Our results, with no important difference in pepsin in nasal secretions and saliva between CRS-patients and controls and no significant difference in pepsin in nasal secretion or saliva samples between CRS patients with proximal reflux or not, may be in line with this, though reservations have to be made due to the limited specificity of the Peptest.

The present study raises important questions about the validity and utility of Peptest as a screening method for extra-esophageal reflux as also discussed by others [22]. One can also question its usefulness in diagnosing GERD in general, since patients with abnormal gastroesophageal reflux had no more positive tests than those without. We strictly followed the recommendations from the manufacturers and found high levels of pepsin in both CRS patients and healthy controls, with even significantly higher levels of pepsin in saliva in controls compared to patients. Accordingly, it appears unlikely that methodological flaws should affect the present results and explain the high levels of pepsin in the control group. As data being the basis for calculation of the specificity of the test varies, and tends to be lower than previously assumed [22], it is relevant to question the practical specificity of the test when used as a diagnostic tool for GERD in upper airway diseases. If Peptest had been a reliable method to diagnose extra esophageal manifestation of GERD, we would also expect more positive tests in patients having proximal reflux as evaluated by 24-h impedance pH monitoring compared to those without. The fact that CRS-patients, even those with severe GER, did not have more pepsin in saliva and nasal secretion than control subjects, supports the speculations about the utility of Peptest in clinical practice. However, we cannot rule out that it may have a role in cases with classical symptoms of reflux, and where test samples can be collected immediately after a reflux episode.

Conclusion

As measured by Peptest, we did not find more pepsin in saliva or nasal secretions in CRS- patients than in healthy controls, nor in those with those with high GerdQ scores or verified proximal reflux, indicating a limited validity of the Peptest as screening tool for GER in CRS.

Abbreviations

CRS= Chronic Rhino Sinusitis, GERD= Gastro Esophageal Reflux Disease, GER= Gastro Esophageal Reflux, VAS= Visual Analog Scale, SNOT-20= Sino Nasal Outcome Test-20, GerdQ= Gastro Esophageal Reflux Disease Questionnaire, BMI= Body Mass Index.

Authorship contribution

EJK, JGH, RO and SKS designed the study. EJK collected the data. EJK analysed and interpreted the data together with JGH, RO, JTK and SKS. EJK wrote the manuscript with assistance from all the authors who also accepted the final version.

Conflict of interest

There is no conflict of interest to report.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

Not applicable.

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