How to Cite:
Buana, A. W., Kristyono, I., & Juniati, S. H. (2022). Nasal pepsin A: Biomarker of laryngopharyngeal reflux in chronic rhinosinusitis. *International Journal of Health Sciences*, *6*(S6), 8599–8608. https://doi.org/10.53730/ijhs.v6nS6.12321

Nasal pepsin A: Biomarker of laryngopharyngeal reflux in chronic rhinosinusitis

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***Abstract*---**Laryngopharyngeal reflux (LPR) can contribute to chronic rhinosinusitis (CRS). Pepsin is believed to be one of the causes of CRS. Pepsin can be a promising biomarker because it is only produced by gastric parietal cells, so it can explain the correlation of CRS with LPR. This study aims to determine the relationship between CRS and LPR based on nasal pepsin examination. The design in this study is a cross-sectional prospective with statistical analysis using the Chi-square method. The research sample were patients with complaints of runny nose and nasal congestion within 12 weeks and met the inclusion and exclusion criteria. All samples were subjected to nasal endoscopy and fiber optic laryngoscope and have Reflux Symptoms Index (RSI) > 13. Specimens from the samples were obtained through the nasal washing method, and pepsin was evaluated using the PepTest lateral flow device. A total of 35 samples in this study consisted of 13 men (37.14%) and 22 women (62.85%). The highest age in the CRS group was the 18-40-year-old group with 16 (64%). A total of 20 (83.3%) CRS patients were positive for pepsin p=0.04 ($p<0.05$). There is a corelation between CRS and LPR based on nasal pepsin examination. The existence of pepsinA in the nasal cavity shows that LPR can be a factor causing CRS.

***Keywords*---**laryngopharyngeal reflux, chronic rhinosinusitis, pepsin, pep test.

Introduction

Chronic rhinosinusitis (CRS) is inflammatory of the mucosa lining the nose and paranasal sinuses with a symptom duration of more than 12 weeks (Fokkens et al., 2012). Research using Taiwanese national data has a twofold relationship between increased risk of chronic rhinosinusitis (CRS) and LPR (Lin, 2015). Laryngopharyngeal reflux (LPR) is the backflow of gastric fluid through the esophagus into the laryngopharynx (Belafsky, 2007). Laryngopharyngeal reflux is currently suspected as one of the causes of CRS onset (Fokkens et al., 2012). Flook and Kumar's study cited in the 2020 European Position Paper on Rhinosinusitis and Nasal Polyp (EPOS) explains that there is insufficient evidence of the outcome of antireflux therapy in adult patients. The study citations also indicate that there is no evidence that acid reflux is a cause of CRS without polyps (Fokken et al., 2012). The incidence of reflux disorders was found to be significantly higher ($p<0.001$) in CRS cases without nasal polyps (Lin, 2015). Research using PepsinA detection in patients with a diagnosis of CRS was shown to have higher levels of positive pepsin in nasal secretions and tissues ($p=0.042$). The presence of pepsin in CRS patients is limited to the epithelial layer or the mucosal cells. The results of this study may indicate a relatively high incidence of LPR in CRS patients (Ren et al., 2017). Other studies have shown that proton pump inhibitor therapy improves reflux symptoms in CRS patients in 79-88% of cases (Beule, 2015). A recent study from Sweden showed that there was a less significant amount of pepsin difference in CRS patients (67.5%) compared to ordinary people (65.4%) through the detection of pepsin in saliva ($p=1.00$) (Katle et al., 2019). Based on these previous studies, this study was conducted to explain the correlation between CRS and LPR based on nasal pepsin examination.

Method

The design of this study used a cross-sectional approach with consecutive sampling methods. The study was conducted from September 2019 to February 2020 in the ORL-HNS Outpatient Unit of the Broncho-Esophagology division and the Rhinology division. Sample examination was carried out in the Clinical Pathology laboratory of Dr. Soetomo general hospital. This research has fulfilled the ethical clearance procedure before conducting the research at Dr. Soetomo general hospital, Surabaya No. 1392/KEPK/VIII/2019).

Patient

The study sample was patients with suspected CRS who came for treatment at the Outpatient Unit of ORL-HNS Dr. Soetomo general hospital, Surabaya, and met the inclusion and exclusion criteria of the study. Inclusion criteria were patients with suspected CRS without polyps who complained of a runny nose or nasal congestion for more than 12 weeks. Patients with LPR symptoms (RSI score >13) and older than 18 years old. Patients are willing to participate in the study by signing informed consent. Exclusion criteria were patients with suspected CRS with polyps, tumors, or severe anatomic abnormalities, as evidenced by nasal endoscopy. The patient took reflux medications in less than 48 hours (antacids, H2 blockers)—smoking and consuming carbonated drinks or alcohol in less than an hour.

Office Procedure

Karl-Storz nasal endoscopy (NE) and fiber optic laryngoscopy (FOL) performed by Rhinologist and Bronchoesophagologist consultant. The diagnosis of CRS follows the EPOS 2020 criteria, while LPR is enforced according to the RSI and RFS questionnaire. Patients who met the inclusion criteria were sampled by nasal irrigation using bidestilate H_2O as much as 0.5 ml from each side using a modified nasal spray. If the examination cannot be carried out on the same day, the specimen must be stored in a refrigerator at a temperature of 5°C for a maximum of 7 days. All specimens were examined by a clinical pathology consultant team and directly supervised by the research team.

Peptest®

Peptest® (RD Biomed Ltd, Cottingham, UK) is an rapid enzyme linked immunoassay for detection of pepsin in saliva (figure 1). This rapid non-invasive test is based on a lateral flow device (LFD) technology. It consists of antibodies to human pepsin and is specific to pepsin A, the isoform secreted only in the stomach (Strugala V *et al.*, 2016).

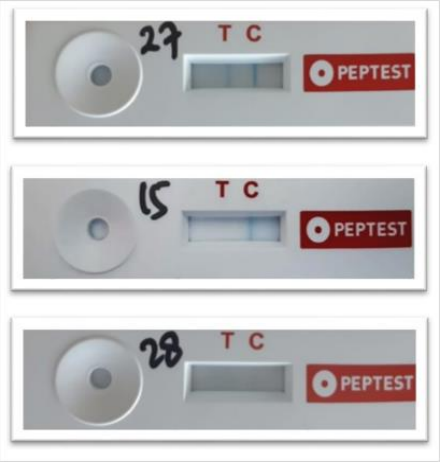

Figure 1. PepTest LFD results; a. positive (T & C) result; b. negative result (C only); c. needs to be repeated.

A control (C) band on the device indicates a correctly performed test and a second line (T) indicates presence of pepsin, defining a positive or negative test. The lower limit for pepsinA detection is 16ng/ mL given by the manufacturer (Strugala V *et al.*, 2016).To the best of our knowledge, Peptest has been used on nasal secretions in some previous studies.

Statistic analysis

The data from the examination were analyzed by using the Chi-Square test. This study uses a significance level of $p<0.05$ with a 95% CI, if it does not meet the requirements it will be tested using the Fisher's Exact Test. Statistical test

analysis using the IBM SPSS Statistics software version 21.0 (IBM Corp., Armonk), NY, USA).

Table 1
Characteristics of Patients

		Group				Amount	
		SSR		non SSR			
		n	%	n	%	n	%
Gender							
	Man	10	40.0	3	30.0	13	37.14
	Woman	15	60.0	7	70.0	22	62.85
	Amount	25	100.0	10	100.0	35	100.00
Age							
	18-40	16	64.0	7	70.0	23	65.71
	41-60	9	36.0	2	20.0	11	31.43
	61-80	0	0.0	1	10.0	1	2.86

Table 2
Reflux symptom index

Reflux symptom index	Number of Patients			
	CRS	%	Non CRS	%
Hoarseness or a problem with your voice	12	48.0	5	50.0
Clearing your throat	25	100.0	10	100.0
Excess throat mucus or post nasal drip	25	100.0	10	100.0
Difficulty swallowing food, liquids or pills	5	20.0	6	60.0
Cough after you ate or lying down	11	44.0	7	70.0
Breathing difficulties or chocking episodes	14	56.0	6	60.0
Troublesome of annoying cough	20	80.0	9	90.0
Sensation of something sticking in your throat or a lump in your throat	25	100.0	10	100.0
Heartburn, chest pain, indigestion, or stomach acid coming up	25	100.0	10	100.0

Table 3
Reflux finding score

Reflux finding score	Number of patients			
	SSR	%	Non SSR	%
Subglottic edema	13	52.0	3	30.0
ventricular obliteration	12	48.0	3	30.0
Erythema and hyperemia	7	28.0	3	30.0
Vocal fold edema	11	44.0	3	30.0
Diffuse laryngeal edema	5	20.0	2	20.0
Posterior commissure hypertrophy	20	80.0	5	50.0
Granulomas/granulation tissue	0	0.0	0	0.0
Thick endo-laryngeal mucus	19	76.0	3	30.0

Results

A total of 35 patients has contributed to this study, consisting of 25 CRS patients and 10 patients diagnosed as non-CRS. The CRS group consisted of 10 males and 15 females, while the non-CRS group consisted of 3 males and 7 females (table 1). The highest age distribution in this study was found at the 18-40 age group as many as 23 (65.71%) patients. While only 1 patient was found at the 61-80 age group, and was included in the non-CRS group (table 1). There were four symptoms experienced by all patients based on the RSI questionnaire, which are clearing your throat, excess throat mucous or postnasal drip, sensations of something sticking in your throat or a lump in your throat, Heartburn, chest pain, indigestion, or stomach acid coming up.

The lowest symptom in the CRS group was difficulty swallowing food, liquids, or pills (5 patients). The lowest complaint in the non-CRS group was hoarseness or a problem with your voice (table 2). The highest RFS finding in CRS cases were posterior commissure hypertrophy in 20 (80%) patients. The second highest complaint was thick endolaryngeal mucusin 19 (76%) patients (table 3). There were no patients who had granuloma/granulation tissue. The pepsinA finding in the CRS group was 20 (83.3%) compare to non-CRS patients 4 (16.7%). In this study, Fisher's correlation test was used to replace the chi-square method because one of the group cells did not meet the minimum sample criteria. There is a significant difference in the number of positive Peptests between patients with CRS and non-CRS. The results of statistical tests using Fisher's Exact Test showed $p=0.04$ ($p<0.05$). Moreover, there is a correlation between positive pepsinA findings in patients with CRS (table 4).

Discussion

Based on the results obtained, the distribution of the most gender groups in CRS patients (table 1) was the female group, 15 (60%) patients. In the male group, 10 (40%) of the total CRS patients. The exact cause of women suffering from CRS is still unclear (Benjamin, 2019). Several factors that cause the high incidence of CRS based on gender differences are anatomy, smoking history, and hormones. Women are more likely to experience complaints mainly due to variations in the anatomical size of the ostium, which are smaller than men. Another reason CRS is the most common in women is passive smoking (Fokkens et al., 2012). Increased immune response in women and the estrogen hormone are also the dominant factors in increasing the inflammatory response (Southwood et al., 2015).

Estrogen factors play a direct role in stimulating parietal cells and the peptic gland without the intervention of the nervous system. This hormonal pathway is mediated by starting from the hypothalamus and anterior pituitary gland, and ACTH affects the adrenal glands to produce cortisone, stimulating parietal cells and peptic glands to produce HCl and pepsin (Belafsky, 2007). The presence of pepsin and the level of acidity have the potential to be biomarkers and the cause of CRS due to LPR (Southwood, 2015). The results of another cohort study by Benjamin et al. (2018) suggest that there is a higher percentage difference

between women (63%) and men in patients with CRS without polyps (Benjamin et al., 2019).

Based on the results, the distribution of most age groups (table 1) is 18-40 years old, with 16 (64%) patients. The results obtained decreased in the 41-60 years age range by 9 (36%) patients. Patients of productive age also experience more complaints due to high exposure to risk factors that cause CRS (Liu et al., 2021). This result is supported by a similar European study which stated that the prevalence rate of CRS will decrease in the age range above 55 years (95%) (Strugala et al., 2016). This study is also supported by Hirsch, et al. (2017) that the decrease in complaints in the USA began to occur at the age of > 59 years as many as 9% of 4,800 CRS patients (Hirsch et al., 2017). The study results found that the four highest complaints were experienced by 35 (100%) of research samples. The complaints are a history of coughing, excessive mucus/runny nose running in the throat, a lumpy sensation in the throat, burning sensation in the pit of the stomach, chest pain, indigestion, and acid regurgitation. The lowest complaint in the CRS group was difficulty swallowing in 5 (20%) of 25 patients. Excess throat mucus or postnasal drip (excess mucus production / runny nose) is one of the symptoms of LPR that is often found in CRS patients.

The direct exposure to reflux of gastric contents is the laryngopharyngeal area and is thought to reach the sinonasal mucosa and cause inflammation in the surrounding mucosa. The inflammatory process will also go through the esophageal-nasal reflex mechanism that occurs indirectly through the vagal reflex. This process will occur along the upper respiratory tract to the sinonasal mucosa, causing vasodilation of blood vessels and extravasation of plasma cells so that the mucosa becomes swollen. The inflammatory process will also affect goblet cells and ciliary dysfunction, leading to excessive secretion and accumulation of secretions. (Sella et al., 2017). The results of this study are in accordance with the research of Ratunanda et al. (2018), which stated that as many as 86 (100%) patients from the total sample experienced excess mucus production or runny nose in the throat in CRS patients with LPR. This study also showed that the mean reflux symptom score (SGR) was 26.43±4.03. The results of the study were also supported by Andriani et al. (2011), proving that a lump in the throat was the most common RSI complaint, 49 respondents (96.08%), while removing mucus in the throat / clearing the throat was found in 43 (83.31%) respondents (Andriani et al., 2011). A similar study in the USA stated that the highest percentage of complaints was post nasal drip, as much as 36.2% of 23700 patients (Hirsch et al., 2017).

Table 4
Analysis of the relationship between CRS and RLF based on nasal pepsin examination

	CRS	%	Non-CRS	%	Amount	%	p
Pepsin +	20	83.3	4	16.7	24	100.0	p=0.04
Pepsin -	5	45.5	6	54.5	11	100.0	
Amount	25	71.4	10	28.6	35	100.0	

* (p<0,05)

The highest distribution of findings based on RFS in CRS cases was posterior commissure hypertrophy in 20 (80%) patients. The second highest complaint was the presence of thick endolaryngeal mucus in 19 (76%) patients. The lowest finding based on RFS in both groups was Granuloma/granulation tissue. Mucosal exposure to refluxate in a non-acidic environment will induce several proinflammatory cytokines and receptors. In a prolonged and repeated state, it will trigger the proliferation and transformation of the epithelium of the exposed organ (Samuels et al., 2010). Mitochondrial damage and mucosal disruption resulting from pepsin exposure can affect the function of mucociliary transport and clearance (Southwood et al., 2015). The results of this study are in accordance with Ratunanda et al. (2018) by showing the mean RFS value is 11.28 ± 1.21 from a minimum score of > 7 and a maximum value of 26. The reflux findings score in this study also shows posterior commissure hypertrophy and mucus Endolaryngitis, a finding in all CRS patients with LPR (Ratunanda et al., 2019). The finding of RFS > 7 was pathologically stated by Siupsinskiene in 2018. The study found a non-significant result (p=0.48) for 64.3% of CRS patients who were proven to have reflux (Siupsinskiene et al., 2018).

In this study, the method of proving the existence of nasal pepsin was used with the lateral flow-based test (PeptestTM, RD BioMed Ltd., UK). This test is easy to perform and can detect pepsin to the lowest limit of 16 ng/mL. This study showed that the positive pepsin findings in the CRS group were 20 (83.3%), with negative pepsin at 5 (45.5%). Non-CSR patients with negative pepsin were 6 (54.5%), while positive for pepsin was 4 (16.7%). The results of statistical tests using Fisher's Exact Test showed p 0.04. These results indicate a significant relationship between CRS and LPR based on nasal pepsin examination. The presence of pepsin in the nasal cavity is the primary variable in this study. The presence of pepsin indicates laryngopharyngeal reflux to the nasal cavity. This theory is based on the principle that pepsin is only produced by gastric parietal cells. The acidity of the refluxed fluid obtained in the extraesophageal organs varies to a pH above 6. The acidity of the mucosal area of the upper respiratory tract is about 7.4. This condition causes the pepsin in the extraesophageal tissue mucosa to be inactive. Gastric reflux fluid containing pepsin has a damaging effect on the mucosa at pH above 4 to 6. The mechanism of pepsin reactivation in extraesophageal tissue occurs in 2 ways. The first mechanism occurs when the subsequent reflux flow is acidic. The second mechanism occurs when the laryngeal epithelium absorbs pepsin into intracellular ones with a lower pH, such as in Golgi apparatus or lysosomes. Pepsin enters the cell through the mechanism of endocytosis (Bardhan et al., 2012).

The epithelium absorbs Pepsin in the inactive form by endocytosis through a competitive receptor media mechanism, then pepsin-filled vesicles will go to the Golgi apparatus. The acidity (pH) of the Golgi body is about 5.5. Golgi bodies are in charge of processing large molecules such as proteins. Cells that absorb inactive Pepsin begin to undergo intracellular damage. Damage starts from the mitochondria to the Golgi apparatus due to the reactivation of Pepsin by an acidic environment. Cell damage was detected within 12 hours after pepsin uptake by the epithelium. Cell damage will appear along with the appearance of markers of oxidative stress and heat shock proteins that are toxic to cells. Further damage will appear along with other markers of cellular oxidative stress and free radicals

(Renn et al., 2017). The results showed that the cells were damaged only once exposed to Pepsin but would survive and remain active within 12 hours. Repeated exposure will cause damaged cells not to last longer (Bardhan et al., 2012).

Exposure to Pepsin in a non-acidic environment will induce several cytokines and proinflammatory receptors. Pepsin will trigger proliferation and epithelial transformation of exposed organs depending on the level and duration of reflux (Samuels et al., 2010). Mitochondrial damage and mucociliary disorders resulting from pepsin exposure can affect the function of mucociliary transport (Southwood etal., 2015). Impaired mucociliary clearance and transport in the ostiomeatal complex will cause changes in the physiology of the paranasal sinus mucosa and cause rhinosinusitis (Fokken et al, 2012). The results of this study are in accordance with Katle et al. (2019), who proved the existence of pepsin in CRS patients using the lateral flow-based test method (PeptestTM, RD BioMed Ltd., UK) as many as 26 (42.6%) compared to 17 controls. (27.9%). There was no significant result (p=0.75) on the presence of nasal pepsin in the morning in CRS patients compared to controls. The study also proved a significant result for the findings of nasal pepsin 2 hours postprandial in 26 (45.6%) CRS patients (p=0.02). Conclusions in this study indicate that the incidence of LPR can be demonstrated in patients with CRS. The rate of nasal pepsin finding was qualitatively significantly higher in the specimens taken 2 hours postprandial (Katle et al., 2019).

The results of this study are also supported by Southwood et al. (2015), who used a combination of nasal lavage pepsin assay with 24-hour dual probe Ph monitoring. This study showed that pepsin was detected in the sinus fluid in 6 (66.6%) CRS patients. This study proves that the reflux component can reach the nasal cavity to enter the paranasal sinuses, and LPR can play a role in the etiology of CRS (Southwood et al., 2015). This study has several limitations, especially the detection of pepsin using Peptest. In this study, only a single sample test was carried out with a 16 ng/ml cut-off point. The study would be better if using spectrophotometry to calculate the quantitative value of pepsin for each specimen and accompanied by three times sampling, both pre and postprandial, with a more significant number of patients.

Conclusion

There is a correlation between CRS and LPR based on human pepsinA examination in the nasal cavity. This can be demonstrated by a significant positive pepsinA examination in the CRS group compared to non-CRS. This study shows that LPR can be one of the factors causing CRS, and LFD can be used to prove the presence of pepsinA in the nasal cavity.

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