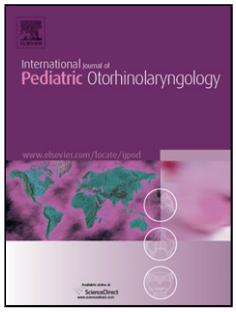

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Author: Giannicola Iannella Giovanni Di Nardo Rocco Plateroti Paolo Rossi Andrea Maria Plateroti Paola Mariani Giuseppe Magliulo

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Investigation of pepsin in tears of children with laryngopharyngeal reflux disease

Giannicola Iannella MD

Giovanni Di Nardo MD *

Rocco Plateroti MD

Paolo Rossi MD *

Andrea Maria Plateroti MD

Paola Mariani PhD °

Giuseppe Magliulo MD

Organi di Senso Department; University" La Sapienza", Viale del Policlinico,151 – 00161, Rome, Italy.

* Department of Pediatrics; University " La Sapienza", Viale del Policlinico,151 – 00161, Rome, Italy.

° Department of General and specialized surgery Paride Stefanini; University "La Sapienza", Viale del Policlinico, 151 – 00161, Rome, Italy.

Mailing list: giannicolaiannella@hotmail.it ; giovanni.dinardo@uniroma1.it ; rocco.plateroti@uniroma1.it ; paolo.rossi@uniroma1.it ; andrea.plateroti@gmail.com ; paola.mariani@uniroma1.it ; giuseppemagliuloorl@yahoo.com

Address correspondence and reprint requests to: Giuseppe Magliulo, M.D., Via Gregorio VII n.80, Rome 00165, Italy; E-mail: giuseppemagliuloorl@yahoo.com ; telephone number: +393388622344 Fax number : +390649976817

Abstract

Objectives: Numerous investigations postulated that Laryngopharyngeal reflux (LPR) is implicated in the pathogenesis of various upper airway inflammatory diseases as sinusitis or dacryostenosis.
The presence of pepsin in tears might be confirmed the presuntive hypothesis of the arrival in the nasolacrimal ducts and precorneal tears film through the laryngopharyngeal reflux of either gastric acid or stomach secretions (pepsin) with inflammatory potentialities.
The aim of this preliminary study was to identify the presence or absence of pepsin in the tears collected from children with a high suspicion of LPR who underwent 24-h pH (MII-pH) monitoring to confirm the disease.
Methods: This study enrolled twenty patients suffering from symptoms of laryngopharyngeal reflux that underwent 24-h multichannel intraluminal impedance (MII)-pH monitoring to confirm the disease. The findings of the study group were compared with those of a control group of patients with negative pH monitoring. The quantitative analysis of human pepsin concentration in the tear samples was performed by ELISA method in both groups.
Results: Four children (20%) of the study group showed pepsin in the tears. All of the subjects belonging to the control group were negative for its presence. No difference differences in the total number of reflux episodes and the number of weakly basic reflux in the pepsin positive patients vs pepsin negative children were present.
Conclusions: 20% of the children with diagnosed LPR showed pepsin in the tears. Our specific investigation might provide information regarding sinusitis or dacryostenosis.

Key Words: Laryngopharyngeal reflux; ph-Metry; Tears; Pepsin; Gastroesophageal reflux; Pediatric;

1. Introduction

Laryngopharyngeal reflux disease (LPRD) is defined as the reflux of gastric and/or duodenal juices (refluxate) beyond the esophagus into larynx, oropharynx, and/or nasopharynx. Although it has been initially considered an extension of gastroesophageal reflux disease (GERD), recently pediatric laryngopharyngeal reflux (LPR) tends to be identified as a unique and distinct disease process[1-4].

Several studies have demonstrated the presence of pepsin and other noxius reflux products, such as bile acids, in middle ear effusion, supporting the existence of a relationship between gastroesophageal reflux (GER) or LPR and otitis media with effusion (OME)[4-15]. This finding implies that these substances are able to reach the middle ear *via* the Eustachian tube, passing through several anatomical structures (larynx, pharynx and rhinopharynx) after their exit from the stomach[4,10,15-19].

Magliulo et[20] al. in 2013 hypothesized that GERD contributes to dacryostenosis and subsequent primary acquired nasolacrimal ducts obstruction as a "prime mover" and so that pepsin could be found in tears. Ascending gastric acid and stomach products might be result in initial edema of the nasolacrimal ducts mucosa which could progress toward chronic inflammation, fibrosis, and, ultimately in a complete nasolacrimal duct obstruction.

The aim of this preliminary study was to identify the presence or absence of pepsin in the tears collected from children with symptoms of laryngopharyngeal reflux disease who underwent 24-h pH (MII-pH) monitoring to confirm the disease.

2. Material and Methods

This prospective study enrolled twenty children (9 males, 11 females; 1-15 years of age, average age 6.6) with a diagnosis of laryngopharyngeal reflux disease between October 2013 and January 2015 at the Pediatrics Department of University 'Sapienza' of Rome. The findings of the study group were compared with those of a control group of patients consisting of twenty normal subjects (10 males and 10 females, age range of 1 to 15 years) who underwent the same diagnostic protocol of the study group (Table 1).

Usually children with symptoms of LPRD, arriving to our Department of Pediatrics, underwent an initial screening by the reflux symptom index (RSI) as developed and validated by Belafsky et al. [21]. In too young children RSI evaluation was made with their parents help.

RSI is a self-conducted survey that includes nine questions with a maximum of 5 points for each question, giving a total of 45 points[22,23]. As suggested by Belafsky et al. [21] any RSI scores above 13 were considered as abnormal.

Children with abnormal RSI underwent Multichannel intraluminal impedance (MII) and pH monitoring to confirm the LPRD.

24-h MII-pH monitoring was performed using an ambulatory system (Sleuth; Sandhill Scientific, Inc; Highland Ranch, CO, USA). The system included a portable data logger with impedance-pH amplifiers and

a MII-pH catheter with an outer diameter of 2.1 mm (6.4-French), containing one pH-measuring electrode and seven impedance sensors, in the form of 4-mm cylindrical ring electrodes. The MII-pH catheter was introduced through the nose and fluoroscopically positioned so that the pH-measuring electrode overlay the third vertebral body above the diaphragm throughout the respiratory cycle. Each participant ate a regular diet and at least 4 h elapsed between each meal.

As no method has been clearly defined to calculate baseline impedance level throughout a 24 h tracing, baseline impedance values were assessed in the most distal channel over the first stable 60-s time period every 4 h. A stable period was identified when no swallowing or bolus or gas reflux was present. Baseline impedance levels during each selected time period were automatically calculated by a specific function (electronic ruler) of the software. Thereafter, the 4-hourly impedance baseline values obtained from the complete tracing were averaged to obtain the mean distal baseline values for the entire recording.

The acid gastroesophageal reflux index (RI), which represents the proportion of the total time of the recording for which the esophageal pH was less than 4.0, was calculated and expressed as a percentage value. RI > 7% was the cut-off value considered for the diagnosis of acid gastroesophageal reflux, according to [24].

All twenty patients enrolled in the study group reported positive detection to the 24-h pH (MII-pH) with an RI> 7, while, all the patients of the children of the study group had values of RI <7.

Besides the following MII-pH variables were analyzed: (1) total number of reflux episodes; (2) number of acid reflux (AR) episodes; (3) number of weakly alkaline episodes (Wal).

All of the patients underwent the withdrawal of the tear sample using a micropipette of clear silicone tube (diameter 0.3 cm, length 2cm and cut 45° degree cut) connected to a small silicone tank (diameter 0.5 cm, length 2 cm) equipped with a 3.5 cm suction tube curved at 0.5 cm with a 30° degree angle. This works by aspiring of tear fluid from the lacrimal lake at inner canthus of the eyelid. All of the samples were stored at -20°C until being analyzed.

The quantitative analysis of human pepsin concentration in the tear samples was performed by ELISA method (commercial pepsin ELISA kit – DRG Inc., Germany). The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of pepsin in mouse serum, plasma and other biological fluids as tears.

Several studies have confirmed as this test is effective to the pepsin evaluation in middle ear effusion or middle ear lavage fluid, however, no study reported the evaluation of human pepsin in tears by this method[1-5,11-13].

The manufacturer claimed as positive for pepsin an ELISA test detection ranged between 1.56-100 ng/mL. However in our case ELISA determination of the human pepsin concentration at 0.0, 2.5, 5, 10 and, 50 ng/ml was performed 10 times according to the manufacturer's instructions to determine the consistency and the lower limit sensitivity of the assay. The standard curve from the average value of the 10 runs had an $R2 = 0.97$; Pepsin (human) at 2.5 ng/ml had a net spectrometer unit increase of 33.5 ± 9.5 % (mean ± SD) over the blank, which was significantly higher than that of the negative control (0.0 ng/ml pepsin consisting only of buffer and reagents) with 9.3± 3.7 % net spectrometer unit increase ($P < 0.05$). Pepsin at 1.5 ng/ml or less had a similar net spectrometer unit increase over the blank as the negative control (0. 0 ng/ml pepsin). Therefore, the empirical pepsin level differentiating positive from negative for pepsin in a tear sample was set at the lower limit of the sensitivity of the assay at 2.5 ng/ml. A patient was defined as pepsin-positive if one of the eye samples had pepsin above 2.5 ng/ml.

All patients guardians gave their written informed consent for the above mentioned tests and to enrolled these patients in the study. This research was performed in accordance with the principles of the Declaration of Helsinki and approved by the local ethics committee of the University " La Sapienza", Rome.

The only descriptive statistical analysis of data was performed due to the limited number of patients in both the study and control group.

3. Results

The percentage of human pepsin in the tears of the study group was estimated in 20% of cases (4 children) all belonging to the group of patients with diagnosis of LPRD. Pepsin was detected in 2 patients younger than <5 years and in one 6 and one 7 year-old patients. Concentration levels of pepsin equal to 3.5, 5.4, 4.0 and 4.2 ng/ml were respectively calculated.

None of the subjects belonging to the control group (negative Negative MII-pH monitoring) reported presence of pepsin in the tears.

Despite the limited number of relevant cases a different pepsin detection about the two groups was evident (Fig. 1). Table 2 summarizes the total number of reflux episodes vs the presence of pepsin in the tears. No difference emerged in two groups because the patients with tears positive to pepsin showed 330 total reflux

episodes (mean value 82.5) vs 1383 total reflux episodes (mean value 86.4) of the group with negative pepsin in tears;

The relationship between the number of acid reflux (AR) episodes and pepsin is described in Table 3. Patients with pepsin positivity showed a lower mean number of acid reflux compared with pepsin negative group (27.5 vs 62).

Table 4 reported the correlation between the number of weakly alkaline reflux episodes and pepsin. Also in this case, no difference emerged between the two groups

4. Discussion

Numerous published investigationshave postulated that LPR and GER are implicated in the pathogenesis of various upper airway inflammatory diseases involving the trachea, larynx, pharynx, paranasal sinuses and middle ear[1-5]. Besides several authors affirmed that while laryngoscopy and pH-monitoring do not have strong predictive value, measurement of pepsin in patients with laryngopharyngeal reflux (LPR) can be considered a potentially reliable diagnostic marker for the correlation of LPRD with oropharynx and nasopharynx diseases[6,11,13,16].

This is also true when the organ is particularly distant from the initial acid or basic reflux such as the middle ear. In 2002, Tasker et al[8] first observed that the majority of their samples taken from children affected by OME contained pepsin/pepsinogen protein even if only 29% were active. Many studies replicated these findings in an effort to establish a definite association between LPR and GER and OME[5-15]. Obviously, their clinical impact was devoted to starting an antireflux therapy with a high level of certainty to avoid unnecessary treament[15-18]. Although no definitive conclusions have yet been drawn, it should be emphasized that the increasing amount of data seems to provide a promising future in this direction.

In any case, it is clear that the route of the reflux to reach the middle ear is particularly circuitous. This consideration a fortiori may account for the potential presence of pepsin in the tears[20]. To reach the pre-corneal film, pepsin has to pass through the nasal fossa, the inferior meatus and the nasolacrimal duct via a mechanism similar to that involved in the pathogenesis of OME[10-15,18]. Remember that the function of the nasolacrimal duct is to collect and drain the tear film into the nasal cavity at the inferior nasal meatus in association with the lacrimal puncta, lacrimal canaliculi and lacrimal sac[25-31]. This physiological

result is assured by its anatomic structure, consisting of many folds of nasal mucosa (the "so-called" valves of Hasner, Foltz or Bochdaalek, Rosenmüller or Huschke, Beraud or Krause, Taillefer and Cruveilhier or Bianchi) devoted to prevent air and mucous from entering the nasolacrimal drainage system. However, since this system of "valves" is not completely competent, air and some secretions may reach the pre-corneal tears film. In fact, in almost 5% of the subjects the Hasner valve appeared incompetent[32]. Moreover, Groell at al[33] demonstrated the presence of air inside the sac and nasolacrimal duct in approximately 29.3% of healthy patients using high-resolution computed tomography.

Pepsin is one of the principal protein-degrading, or proteolytic, enzymes in the digestive system, formed in the stomach after conversion of pepsinogen I. Our investigation was specifically designed to measure pepsin presence and concentration in the tears. This was based upon the presumptive hypothesis of its arrival in the pre-corneal tears film through the nasolacrimal duct after reaching the nasopharynx through the reflux attacks [13]. Our empirical normative data of the pepsin level differentiating positive from negative in a tear sample was set at the lower limit of the sensitivity of the assay at 2.5 ng/ml. In our study four children (20%) with positive diagnosis of LPR showed pepsin above this limit. To confirm the association between LPR and pepsin in tears should be note that all of the subjects belonging to the control group (negative Negative MII-pH monitoring) were negative for pepsin presence in tears. Obviously should be considered that this data merits further study in larger series of patients.

The level of pepsin in the four positive children appears particularly low (mean value 4.2 ng/ml) and, likely, insufficient for determining inflammatory activity in the nasolacrimal duct and in the precorneal conjunctival cavity.

Although these data we can't exclude a potential endogen pepsin production or its coming from plasma. However it is reasonable to consider that in case of local production or plasma migration it is difficult that the pepsin values exceed the concentration of 2.5 ng/ml as reported in or four patient with diagnosis of LPR.

Despite the presence of pepsin in the tears correlates positively with the LPRD, no correlation in terms of both total number and weakly alkaline reflux episodes appeared in our clinical study. However patients with pepsin positivity showed a lower mean number of acid reflux compared with pepsin negative group (27.5 vs 62).

To our knowledge, this is the first study that confirms the presence of pepsin in the tears and the association with LPR. The evaluation of pepsin in children with laryngopharyngeal reflux disease might

provide information regarding chronic inflammation of nasopharynx and nasolacrimal ducts which might lead versus chronic sinusitis, dacryostenosis or OME.

This study demonstrated that pepsin can be detected in tears. It might mean that reflux probably can reach nasolacrimal duct and tears and in some patients with simultaneous pathology of lacrimal duct draining function might be cofactor of inflammatory changes. However our observation of the ascending gastric enzymes through LPR merits more in-depth research to assess its incidence and its potential clinical impact. Further investigation is underway to evaluate this specific topic in order attempt to enhance the sensitivity and specificity of this assay.

5. Conclusion

1. 20% of the children with diagnosed LPRD showed pepsin in the tears.
2. All of the subjects belonging to the control group were negative for its presence.
3. Pepsin in the tears does not correlate with total number and types of reflux episodes.

Conflicts of interest: none

Table 1: Patients' characteristics and ELISA pepsin evaluation in tears

	N. of patients	Sex	Average age (years)	Positive pepsin in tears
LPRD group (Positive MII-PH monitoring)	20	11 Male 9 Female	6.6	4 (20%)
Control group Negative MII-pH monitoring)	20	10 Male 10 Female	6.9	0

Laryngopharyngeal reflux disease (LPRD); Multichannel intraluminal impedance (MII)

Table 2: Incidence with respect to the tototal number of reflux episodes.

Patients	Total number of reflux episodes	Mean value
Pepsin + (n=4)	330	82.5
Pepsin - (n=16)	1383	86.4

Table 3: Incidence with respect to the number of acid reflux episodes.

Patients	Number of acid reflux episodes	Mean value
Pepsin + (n=4)	110	27.5
Pepsin - (n=16)	992	62

Table 4: Incidence with respect to the number of weakly alkaline reflux episodes.

Patients	Number of weakly alkaline reflux	Mean value
Pepsin + (n=4)	6	1.5
Pepsin - (n=16)	52	3.2

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FIGURE CAPTIONS

Fig. 1: Concentration of pepsin in tears (ng/ml) in LPRD and control groups.