

Gastric Pepsin in Middle Ear Fluid of Children With Otitis Media: Clinical Implications

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Gastroesophageal reflux and extraesophageal reflux have been postulated to be involved in the pathogenesis of otitis media. This is supported by recent studies revealing the presence of gastric pepsin in the middle ear space of children with otitis media but not in control patients without otitis media. Reflux's role in otitis media appears to be most pronounced in younger children and those with purulent effusions.

Introduction

Otitis media is the second most common reason for children to visit the pediatrician [1]. This disease's incidence appears to be on the rise; thus, it is vital to elucidate its pathophysiology to allow for more effective prevention and treatment [2–5]. Multiple risk factors for otitis media in children have been identified, including adenoid hypertrophy, pacifier use, smoke exposure, presence of allergy, order of birth, craniofacial anomalies, ethnicity, age, group child care, season of the year, lack of breastfeeding, and genetic predisposition [4]. However, the role of gastroesophageal and extraesophageal reflux in this disease only recently has been recognized. The discovery of gastric pepsin in middle ear fluid has provided direct evidence for this relationship and opens new opportunities to expand understanding of extraesophageal reflux's role in this common childhood disease.

Gastroesophageal reflux occurs in approximately two thirds of infants at 4 months of age but decreases as the child matures [6–8]. Once these reflux events cross a biologic threshold, pathologic changes are produced in the esophagus or upper aerodigestive tract and are termed *gastroesophageal reflux disease* (GERD) or *extraesophageal*

reflux disease (EORD) [9,10,11•]. As otitis media is produced by eustachian tube dysfunction [12,13], EORD is likely to produce inflammation of the tubal mucosa and surrounding structures of the nasopharynx [12,14,15]. Alterations in native mucosal defense mechanisms, together with mucosal inflammation, have been shown to result from reflux events [16•].

Five separate lines of clinical and experimental evidence have implicated GERD/EORD in otitis media: 1) clinical evidence for GERD/EORD producing upper aerodigestive tract pathology other than otitis media, 2) animal models of GERD/EORD and otitis media, 3) clinical evidence for GERD/EORD producing otitis media, 4) studies of pepsin's role in upper aerodigestive tract pathology other than otitis media, and 5) studies of pepsin in otitis media pathophysiology. In this review, we focus on studies involving the detection of pepsin in the middle ear of children with otitis media and its potential role in the disease.

Pathophysiology of EORD in Otitis Media

Extraesophageal reflux may contribute to eustachian tube dysfunction and induce otitis media in several ways. Refluxate may induce mucosal inflammation through production of an acidic environment, toxicity of conjugated bile acids, and osmotic damage [14,15]. However, the consensus is that mucosal damage is principally mediated by proteolytic activity from pepsin [17•]. Radiographic evidence has shown that fluid can traverse the eustachian tube and enter the middle ear cleft from the nasopharynx during swallowing [18]. The ciliated respiratory epithelium that lines the human eustachian tube can undergo ciliostasis and subsequent disruption of mucociliary clearance below a pH of 4 [19–21]. Acid and nonacid EORD theoretically can lead to functional eustachian tube obstruction due to impaired mucociliary clearance, incomplete opening, and true anatomic obstruction from mucosal edema and adenoid hyperplasia. This leads to impaired pressure equalization with persistent negative pressure, a condition that has been shown to result in middle ear effusions [22,23].

Animal models have demonstrated EORD's effects on eustachian tube function. Exposure of the nasopharyngeal orifice of the eustachian tube to simulated reflux in rats has resulted in persistent abnormalities in pressure equalization [15,22], and a gerbil model of EORD demonstrated that refluxate can readily penetrate the eustachian tube and enter the middle ear cleft [24].

Anecdotally, we have encountered pediatric patients with chronic, refractory tube otorrhea who have responded to antireflux medications. Clinical reports have validated this association linking EORD to otitis media. Evidence of distal and proximal reflux monitored by using pH probe has been documented in patients with refractory discharging ears, and they have shown a positive response to medical treatment of GERD [8]. pH probe evidence for GERD is found in up to 56% of children with chronic serous and recurrent otitis media. In children with evidence of pharyngeal and esophageal reflux on double-probe pH testing, a 10-fold and 5.3-fold greater risk, respectively, of developing middle ear fluid has been documented compared with children with normal pH studies [25,26].

Pepsin as a Marker of EORD in Otitis Media

Although other types of evidence have implicated EORD in otitis media, it was not until gastric pepsin was detected and confirmed in the middle ear cleft of patients with otitis media that this association's scientific validity was established. Pepsin's principal role in the stomach is to digest proteins at low pH. Pepsin is produced by the chief cells of the stomach as the gastric proteolytic proenzyme pepsinogen [27,28]. It is converted to active form at pH below 4.0 and uses aspartic acid residues in its active center to bind the substrate [27]. At least seven proteolytic enzymes are produced in the human stomach, with the isoenzyme pepsinogen/pepsin A predominating, although a significant amount of pepsinogen/pepsin C is also produced there, accounting for 30% to 40% of total enzymatic activity of gastric pepsin [17,28].

Studies of pepsin in otitis media have revealed four important facts: 1) pepsin is consistently detected in the middle ear cleft of patients with otitis media at the dilute concentrations expected for reflux through the eustachian tube, 2) it is not concentrated from serum or endogenously produced in the middle ear, 3) it is associated with GERD in children, and 4) it is found at a statistically higher rate in patients with otitis media than in controls without otitis media. The four points are discussed in the following sections.

Detection of pepsin in the middle ear

Tasker et al. [29] published the first report on pepsin in middle ear effusions in 2002. Fifty-four children 2 to 8 years old undergoing myringotomy for mucoid middle ear effusion were tested for pepsin. Forty-five (83%) of the

effusions were positive for pepsin using direct enzyme-linked immunosorbent assay (ELISA) with an antibody to porcine pepsinogen/pepsin. The mean concentration of pepsin/pepsinogen was 36.6 mg/L. The proteolytic activity, measured at pH 2.0, was detected in 24% of samples. In a subsequent report using the same methods, Tasker et al. [30] evaluated 65 effusions from children with findings similar to those of the first study. They also demonstrated that the antibody used in their ELISA assay was not found experimentally to be cross-reactive with albumin or gammaglobulin, but fibrinogen did show cross-reactivity, giving an interference of up to 12%. The authors suggested that the discrepancy between the percentages of pepsin-positive samples on ELISA versus the enzymatic assay may be explained by the fact that the pH of effusion samples was so high that most pepsin would be irreversibly inhibited.

To verify this unexpectedly high rate of pepsin in the middle ear, we studied middle ear aspirates from 152 children with the average age of 2.8 years undergoing myringotomy with tube placement [31]. The enzymatic assay developed by Krishnan et al. [32] was used and modified to increase the lower limit of sensitivity from 250 ng/mL to 12.5 ng/mL. A total of 14% of the patients in the study demonstrated pepsin activity above the detectable level of 12.5 ng/mL, with an average concentration of 96.6 ng/mL (range, 13–687 ng/mL). The prevalence of pepsin-positive ears in our study was significantly less than the rate found in the previous reports [29,30]. We performed direct ELISA and Western blot using the same commercial antibody that was used in the previous studies [29,30] in selected samples. Our data showed that more than 90% of samples were positive for pepsin using ELISA assay, but Western blot results revealed that the antibody, in addition to recognizing pepsin, also recognized a protein with a very strong signal at a higher molecular weight. This protein was also observed in normal serum (He et al., unpublished data). This unknown protein was observed in most of our tested samples because middle ear fluid samples are typically contaminated with blood. The recognition of this contaminant by the polyclonal antibody likely contributed to the high pepsin false-positive rates reported when testing with ELISA using this antibody. The difference in the pepsin-positive rates found in our enzymatic method and those found in Tasker et al.'s [29,30] studies also may have been due to differences in substrates used, sample size, or population studied.

In the most recent report on pepsin and otitis media, Crapko et al. [33] used an antipepsin peptide antibody to perform Western blot analysis of middle ear effusions aspirated from 20 pediatric patients at the time of tympanostomy tube placement. This assay had a lower sensitivity limit of 0.05 ng/mL of pepsin. Twelve of 20 patients (60%) had detectable pepsin in the effusion, with a concentration range of 80 to 1000 ng/mL. A band at higher molecular weight than pepsin was observed in the

samples on Western blot. Based on its molecular weight, it is likely to be the same band we observed in our study. Mass spectroscopy revealed it to be serum albumin [33]. This report is consistent with the concentration range of pepsin reported in our current study [31]. The higher rate of pepsin in the middle ear in the Crapko et al. [33] study is probably due to better sensitivity of their antibody (0.05 ng/mL vs our sensitivity of 12.5 ng/mL). In another study, using a sandwich ELISA assay to human pepsinogen I, Lieu et al. [34] showed that 77% of patients were positive, and the proteolytic enzymatic assay revealed that 73% of patients were positive. In two separate studies, Sone et al. [35,36] used an immunoassay to detect pepsinogen in the middle ear fluid of adult patients with otitis media and found that pepsinogen concentrations ranged from 9 to 3600 ng/mL.

Antonelli et al. [37] studied a group of children 2 to 16 years old with untreated post-tympanostomy tube otorrhea. Twenty-four samples of otorrhea were tested using a commercial ELISA for pepsinogen I. Eight of the samples were positive above the test sensitivity of 1.2 µg/L, ranging in concentration from 2 to 17 µg/L. These levels were not above the negative control serum. They used a different, more specific antibody in their ELISA to pepsinogen I than the antiporcine pepsin used by Tasker et al. [29,30], so to explain the discrepancy in results, the authors pointed out that post-tympanostomy tube otorrhea contains bacteria and a variety of proteolytic enzymes that may degrade the pepsin in the sample. They also suggested that the assay used by Tasker et al. [29,30] might have cross-reacted with proteins other than pepsin/pepsinogen, leading to a high false-positive rate. However, the small sample size could also be the reason for failing to detect pepsin.

In an abstract originally presented at the American Academy of Otolaryngology Head and Neck Surgery annual meeting, Bates et al. [38] reported on a Western blot assay to assess pepsin in the middle ear fluid of children less than 4 years old with chronic otitis media with effusion. Pepsin was detected using Western blot with an antibody specific for human pepsin. None of the 27 effusions from 16 children were reported to show detectable pepsin (detection limit, 20 ng/mL), and no enzymatic activity was found in the samples tested. Although the authors offered no explanation for the discrepancy between these findings and prior studies, they stated that the negative results did not rule out an etiologic role of gastroesophageal reflux in chronic otitis media with effusion [38]. Certainly, the challenges in handling protein-rich fluid in these assays may explain this discrepancy. Apparently, the true incidence of detecting pepsin in the middle ear of children with otitis media needs further investigation.

Pepsin in the middle ear space has gastric origin

Low levels of pepsinogen/pepsin can be detected with very sensitive ELISA methods; thus, one concern is that pepsin found in the middle ear space could be concentrated

from serum or endogenously produced in the middle ear cleft. In a study of middle ear aspirates for pepsin, Tasker et al. [30] found that the samples had a mean albumin concentration equivalent to that of serum. However, the middle ear pepsin concentrations far exceeded serum pepsin concentrations, indicating that the pepsin had not been concentrated in the middle ear cleft from serum [30]. Several other studies have duplicated this finding using a comparison of pepsin, albumin, and fibrinogen levels of ear samples with serum [33,39]. Lieu et al. [34] found the gene encoding for pepsinogen I/A in two samples of gastric mucosa via reverse transcriptase polymerase chain reaction but not in separate discarded specimens from mastoid mucosa [34]. This indicated that the pepsin found in the middle ear cleft could not be endogenously produced in the middle ear mucosa.

It was demonstrated recently that pepsinogen C, the same protein produced in the stomach, is also expressed in lung epithelial cells [40]. This raises the question of whether pepsinogen C is also expressed in the middle ear. If so, the total enzymatic activity measured at a low pH would include pepsin A and pepsin C activity, resulting in overestimation of pepsin in middle ear fluid or perhaps false pepsin positivity. However, two lines of evidence indicate otherwise. First, in some pepsin studies, pepsin detection has been verified by using immunoassay with an antibody specific to pepsin A [31,33,34]. Although pepsin A and pepsin C are from the same gene family, they share only 50% identity in amino acid sequence and can be distinguished by immunoassay [41]. Pepsin A has never been demonstrated to be produced in an extragastric location. In a preliminary investigation, we have detected pepsin C in middle ear fluid by Western blot using an antibody specific to human pepsin C. In the stomach, 60% to 70% of acidic protease activities are from pepsin A, and 30% to 40% from pepsin C and other minor acidic proteases. Therefore, detection of pepsin C in the middle ear is expected if gastric fluid gains access to the middle ear space. We found the ratio of pepsin A to pepsin C in middle ear fluid to be very similar to that observed in gastric fluid, indicating that pepsin C detected in middle ear fluid has gastric origin, as the ratio of pepsin A to pepsin C would likely be lower if pepsin C were endogenously produced in the middle ear space (He et al., unpublished data).

Association of extragastric pepsin detection with GERD or EORD

Although more studies are emerging to investigate the incidence of pepsin in the middle ear, few have been designed to analyze the association of its detection with GERD or EORD. In 2005, Lieu et al. [34] reported finding pepsin/pepsinogen in effusion samples from 22 children (age 1–6.5 years) with otitis media and attempted to correlate these observations with a questionnaire of GERD symptoms. Patients with a medical history predisposing them to GERD or “airway or respiratory disease that might be

associated with GER” were excluded. Symptoms of reflux were no more prevalent in the pepsin-positive children in the study than in normal children. They concluded that these patients, although showing evidence of EORD reaching the middle ear, do not necessarily complain of EORD symptoms. The realization that clinical symptoms of GERD in children are not adequate predictors of significant EORD has prompted further studies. This has not been thoroughly investigated in children, but data on adults have been produced. Sone et al. [35] tested pepsinogen in the middle ear fluid of 61 adult patients with otitis media with effusion. The patients were asked to fill out a questionnaire regarding reflux symptoms. The proportion of patients with pepsinogen concentrations greater than 500 ng/mL was higher in those with positive GERD as determined by the questionnaire. Additionally, four patients with pepsinogen concentrations higher than 1000 ng/mL had repeat aspirations of the middle ear fluid after treatment with a proton pump inhibitor for an unspecified length of time. The pepsinogen levels decreased substantially in all four patients. The authors also reported that in unpublished data, effusions from patients with “defined” causes of middle ear effusion other than GERD showed pepsinogen levels below 18 ng/mL. This clear association between GERD and detection of pepsin in the middle ear in adults has not been found in pediatric populations.

El-Fattah et al. [39] reported on 31 children with otitis media who had undergone 24-hour dual-probe pH testing, and pepsin was tested using direct ELISA in 17 children (mean age, 6.2 years) who underwent myringotomy with tube placement. Twenty-two children showed a positive pH probe study, indicating high prevalence of laryngopharyngeal reflux in otitis media. All 17 middle ear samples were pepsin-/pepsinogen-positive, and the pepsin levels in the middle ear effusions were significantly correlated with proximal (laryngopharyngeal) reflux episodes. In children with laryngopharyngeal reflux, 51% had normal distal esophageal acid exposure time, suggesting that EORD—and not GERD in isolation—predisposes to otitis media with effusion. The authors concluded that EORD, not isolated GERD, is necessary to predispose to otitis media with effusion in children. The high prevalence of pepsin in the middle ear samples is somewhat suspect, as the antibody used in the study was the same one that has been shown to cross-react with serum protein on Western blot. The association of subjective GERD symptoms with detection of pepsin in the middle ear cleft is still an open question in children.

In our previous work involving 152 children with otitis media, we found no association between clinical history of GERD, allergy, or asthma and positive pepsin assay [31]. In our most recent work examining 509 children, detection of pepsin in the middle ear was not associated with GERD by medical history or by objective testing or with other clinical conditions such as allergy and asthma [11•]. This may be due to a true lack of association between the

medical history and otitis media or due to the inaccuracy of the clinical history. The latter explanation seems plausible given the small percentage of patients with objectively documented evidence of GERD, allergy, or asthma. This is particularly important with respect to EORD, as clinical history of reflux has been demonstrated repeatedly to lack correlation with objective testing (pH/impedance probe testing) in this age group [42,43].

Importance of a control group in studies of pepsin and otitis media

Because a strong association between detection of pepsin in the middle ear of children with otitis media and clinical symptoms of GERD/EORD had not been demonstrated, doubts existed regarding the significance of gastric pepsin in otitis media. It was entirely possible that aspirating gastric fluid into the middle ear space was a random but normal physiologic event in children regardless of their clinical condition. As otitis media pathophysiology was recognized to be a complex and likely multifactorial process, EORD’s role in otitis media required evidence from a suitable control group without otitis media. This had not been addressed in previous studies. Therefore, we designed and executed a study with a large sample size that involved the prospective recruitment of 509 pediatric patients (age 2.7 ± 2.4 years) undergoing myringotomy with tube placement for recurrent acute otitis media and/or otitis media with effusion. We also included 64 children (age 4.8 ± 4.2 years) with no documented history of otitis media undergoing cochlear implantation as a suitable control group [11•]. Our previously described enzymatic method was used to measure pepsin activity in middle ear samples, and Western blot was carried out in selective samples to verify the results. The incidence of pepsin in the patients with otitis media was significantly higher (20%) than that in the control patients (1.5%). The control group was older and by definition had a “dry” middle ear space. When the age and ear status were matched between the two groups, the pepsin positivity was still significantly different ($P < 0.05$). This was the first study that included a control group with adequate sample size to examine the association of GERD with otitis media and provided compelling evidence that the detection of pepsin in the middle ear space of children is associated with otitis media.

Another novel finding in our study [11•] was that patients with dry ears were 13% pepsin-positive, significantly lower than patients with middle ear effusions at the time of tube placement. Patients with purulent effusions had the highest rate of positive pepsin assay at 47%; the difference was still significant even when it was compared with an age-matched patient or age-matched mucoid/serous effusion group. Previous studies have demonstrated that the concentration of inflammatory mediators such as interleukin-8 differs among subtypes of middle ear effusions [44,45]. Different types of middle ear effusion may represent different degrees of middle ear

inflammation or may reflect variable degrees of eustachian tube dysfunction. We could not draw conclusions in our study group because objective tests of eustachian tube function were not performed. The compelling question raised is whether the presence of pepsin in the middle ear escalates the degree of inflammation or simply reflects the degree of patency of the eustachian tube [44,45]. This requires further study.

We also discovered in our study that the highest rate of pepsin-positive patients was found in younger children. Children less than 1 year old had a pepsin-positive rate of 31%, which is significantly higher than that of other age groups. This age-dependent pattern remains in different subgroups in which other factors are controlled. This suggests that EORD may be an important initiator of otitis media in younger children. That GERD is very common in young children but decreases as they age presents the possibility that what had been deemed *physiologic reflux* may not be so benign but may play a role in otitis media. Clearly, multiple factors are involved in otitis media development, but our data clearly show that EORD is a factor in a subset of children. It is possible that in susceptible children, EORD may be the sole cause of otitis media, but further work is required to enable identification of these patients.

Conclusions

There is no doubt that gastric pepsin can reach the middle ear space and be detected in patients with otitis media. Absence of gastric pepsin in control patients without otitis media provides compelling evidence that reflux plays a role in this disease's pathophysiology. That pepsin is more prevalent in the middle ears of younger children with otitis media and in those with purulent effusions may have implications regarding the mechanism of action of reflux on eustachian tube function. Future work will focus on elucidating these mechanisms.

Disclosures

No potential conflicts of interest relevant to this article were reported.

References and Recommended Reading

Papers of particular interest, published recently, have been highlighted as:

- Of importance
 - Of major importance
1. Lubianca Neto JF, Hemb L, Silva DB: Systematic review of modifiable risk factors for recurrent acute otitis media in childhood. *J Pediatr (Rio J)* 2006, 82:87–96.
 2. Hing E, Middleton K: National hospital ambulatory medical care survey: 2001 outpatient department survey. *Adv Data* 2003, 338:1–26.
 3. Berman S, Byrns PJ, Bondy J, et al.: Otitis media-related antibiotic prescribing patterns, outcomes, and expenditures in a pediatric Medicaid population. *Pediatrics* 1997, 100:585–592.
 4. Bluestone CD: Studies in otitis media: Children's Hospital of Pittsburgh-University of Pittsburgh Progress Report-2004. *Laryngoscope* 2004, 114(Suppl 105):1–26.
 5. Rosenfeld RM, Culpepper L, Doyle KJ, et al.: Clinical practice guideline: otitis media with effusion. *Otolaryngol Head Neck Surg* 2004, 130(Suppl 5):S95–S118.
 6. Nelson SP, Chen EH, Syniar GM, Christoffel KK: Prevalence of symptoms of gastroesophageal reflux during infancy. A pediatric practice-based survey. Pediatric Practice Research Group. *Arch Pediatr Adolesc Med* 1997, 151:569–572.
 7. Gibson WS, Cochran W: Otagia in infants and children: a manifestation of gastroesophageal reflux. *Int J Pediatr Otorhinolaryngol* 1994, 28:213–218.
 8. Poelmans J, Tack J, Feenstra L: Prospective study on the incidence of chronic ear complaints related to gastroesophageal reflux and on the outcome of antireflux therapy. *Ann Otol Rhinol Laryngol* 2002, 111:933–938.
 9. McGuirt WF: Gastroesophageal reflux and the upper airway. *Pediatr Clin North Am* 2003, 50:487–502.
 10. Burton DM, Pransky SM, Katz RM, et al.: Pediatric airway manifestations of gastroesophageal reflux disease. *Ann Otol Rhinol Laryngol* 1992, 101:742–749.
 11. O'Reilly RC, He Z, Bloedon E, et al.: The role of extra-esophageal reflux in otitis media in infants and children. *Laryngoscope* 2008, 118(Suppl 116):1–9.
- Largest clinical study to date on otitis media and EORD and the only one to include a control group; the online appendix gives a comprehensive review of background to this problem.
12. Bluestone CD: *Eustachian Tube: Structure, Function, and Role in Otitis Media*. London: BC Decker; 2005.
 13. Bluestone CD, Klein JO: Otitis media and eustachian tube dysfunction. In *Pediatric Otolaryngology*. Edited by Bluestone CD, Stool SE, Alper SM, et al. Philadelphia: Saunders; 2003:474–685.
 14. Heaven SB, Hardy SM, White DR, et al.: Transient inflammation and dysfunction of the eustachian tube secondary to multiple exposures of simulated gastroesophageal refluxant. *Ann Otol Rhinol Laryngol* 2001, 110:928–934.
 15. Heaven SB, Hardy SM, White DR, et al.: Function of the eustachian tube after weekly exposure to pepsin/hydrochloric acid. *Otolaryngol Head Neck Surg* 2001, 125:123–129.
 16. Johnston N, Dettmar PW, Lively MO, et al.: Effect of pepsin on laryngeal stress protein (Sep70, Sep53, and Hsp 70) response: role in laryngopharyngeal reflux disease. *Ann Otol Rhinol Laryngol* 2006, 115:47–58.
- Excellent study of the effects of pepsin exposure on mucosa of the upper aerodigestive tract with ramifications for all aspects of EORD.
17. Roberts NB: Review article: human pepsins—their multiplicity, function and role in reflux. *Aliment Pharmacol Ther* 2006, 24(Suppl 2):2–9.
- Concise but thorough review of pepsins.
18. Wittenborg MH, Neuhauser EB: Simple roentgenographic demonstration of eustachian tubes and abnormalities. *Am J Roentgenol Radium Ther Nucl Med* 1963, 89:1194–1200.
 19. Holma B, Lindegren M, Andersen JM: pH effects on ciliomotility and morphology of respiratory mucosa. *Arch Environ Health* 1977, 32:216–226.
 20. Ohashi Y, Nakai Y, Koshimo H, Esaki Y: Ciliary activity in the in vitro tubotympanum. *Arch Otorhinolaryngol* 1986, 243:317–319.
 21. Park K, Bakaletz LO, Coticchia JM, Lim DJ: Effect of influenza A virus on ciliary activity and dye transport function in the chinchilla eustachian tube. *Ann Otol Rhinol Laryngol* 1993, 102:551–558.

22. Butehorn HF, Downs BW, Prazma J, Pillsbury HC: **Acute gastroesophageal reflux and eustachian tube function [abstract]**. *ARO Abstracts of the Twenty-Second Midwinter Research Meeting*. Mount Royal, NJ: Association for Research in Otolaryngology; 1999:199.
23. Moody SA, Alper CM, Doyle WJ: **Daily tympanometry in children during the cold season: association of otitis media with upper respiratory tract infections**. *Int J Pediatr Otorhinolaryngol* 1998, 45:143–150.
24. Sudhoff H, Bucker R, Groll C, et al.: **Tracing of gastric reflux into the middle ear in a Mongolian gerbil model**. *Otol Neurotol* 2006, 28:124–128.
25. Rozmancic V, Velepich M, Ahel V, et al.: **Prolonged esophageal pH monitoring in the evaluation of gastroesophageal reflux in children with chronic tubotympanic disorders**. *J Pediatr Gastroenterol Nutr* 2002, 34:78–80.
26. Keles B, Ozturk K, Gunel E, et al.: **Pharyngeal reflux in children with chronic otitis media with effusion**. *Acta Otolaryngol* 2004, 124:1178–1181.
27. Martinsen TC, Bergh K, Waldum HL: **Gastric juice: a barrier against infectious disease**. *Basic Clin Pharmacol Toxicol* 2005, 96:94–102.
28. Jones AT, Roberts NB: **Peptide maps of five human pepsin isoenzymes and other aspartic proteinases**. *J Chromatogr* 1993, 646:207–212.
29. Tasker A, Dettmar PW, Panetti M, et al.: **Reflux of gastric juice and glue ear in children**. *Lancet* 2002, 359:493.
30. Tasker A, Dettmar PW, Panetti M, et al.: **Is gastric reflux a cause of otitis media with effusion in children?** *Laryngoscope* 2002, 112:1930–1934.
31. He Z, O'Reilly RC, Bolling L, et al.: **Detection of gastric pepsin in the middle ear fluid of children with otitis media**. *Otolaryngol Head Neck Surg* 2007, 137:59–64.
32. Krishnan U, Mitchell JD, Messina I, et al.: **Assay of tracheal pepsin as a marker of reflux aspiration**. *J Pediatr Gastroenterol Nutr* 2002, 35:303–308.
33. Crapko M, Kerschner JE, Syring M, Johnston N: **Role of extra-esophageal reflux in chronic otitis media with effusion**. *Laryngoscope* 2007, 117:1419–1423.
34. Lieu JE, Muthappan G, Uppaluri R: **Association of reflux with otitis media in children**. *Otolaryngol Head Neck Surg* 2005, 133:357–361.
35. Sone M, Yamamuro Y, Hayashi H, et al.: **Otitis media in adults as a symptom of gastroesophageal reflux**. *Otolaryngol Head Neck Surg* 2007, 136:19–22.
36. Sone M, Yamamuro Y, Hayashi H, et al.: **Prediction of gastroesophageal reflux in otitis media with effusion in adults**. *Acta Otolaryngol* 2007, 127:470–473.
37. Antonelli PJ, Lloyd KM, Lee JC: **Gastric reflux is uncommon in acute post-tympanostomy otorrhea**. *Otolaryngol Head Neck Surg* 2005, 132:523–526.
38. Bates DD, Lively M, Koufman J, et al.: **Pepsin not detected in middle ear effusions in children [abstract]**. *Otolaryngol Head Neck Surg* 2006, 135(2 Suppl):99.
39. El-Fattah A, Maksoud G, Ramadan A, et al.: **Pepsin assay: a marker for reflux in pediatric glue ear**. *Otolaryngol Head Neck Surg* 2007, 136:464–470.
40. Foster C, Aktar A, Kopf D, et al.: **Pepsinogen C: a type 2 cell-specific protease**. *Am J Physiol Lung Cell Mol Physiol* 2004, 286:L382–L387.
41. Taggart RT, Cass LG, Mohandas TK, et al.: **Human pepsinogen C (progastricsin): isolation of cDNA clones, localization to chromosome 6, and sequence homology with pepsinogen A**. *J Biol Chem* 1989, 264:375–379.
42. Gupta SK, Hassall E, Chiu YL, et al.: **Presenting symptoms of nonerosive and erosive esophagitis in pediatric patients**. *Dig Dis Sci* 2006, 51:858–863.
43. Bauman NM, Bishop WP, Sandler AD, Smith RJ: **Value of pH probe testing in pediatric patients with extraesophageal manifestations of gastroesophageal reflux disease: a retrospective review**. *Ann Otol Rhinol Laryngol Suppl* 2000, 184:18–24.
44. Nassif PS, Simpson SQ, Izzo AA, Nicklaus PJ: **Interleukin-8 concentration predicts the neutrophil count in middle ear effusion**. *Laryngoscope* 1997, 107:1223–1227.
45. Chung MH, Choi JY, Lee WS, Yoon JH: **Compositional difference in middle ear effusion: mucous versus serous**. *Laryngoscope* 2002, 112:152–155.