The Role of Gastric Pepsin in the Inflammatory Cascade of Pediatric Otitis Media

Robert C. O’Reilly, MD; Sam Soundar, PhD; Dalal Tonb, ScD; Laura Bolling, BS; Estelle Yoo, MD; Tracey Nadal, BS; Christopher Grindle, MD; Erin Field, PA; Zhaoping He, PhD

IMPORTANCE Otitis media is characterized as an ongoing inflammation with accumulation of an effusion in the middle ear cleft. The molecular mechanisms underlying the pathogenesis, particularly the inflammatory response, remain largely unknown. We hypothesize that aspiration of gastric contents into the nasopharynx may be responsible for the initiation of the inflammatory process or aggravate a preexisting condition.

OBJECTIVE To investigate the correlation of gastric pepsin A with inflammatory cytokines, bacterial infection, and clinical outcomes.

DESIGN, SETTING, AND PARTICIPANTS Prospective study of 129 pediatric patients undergoing myringotomy with tube placement for otitis media at a tertiary care pediatric hospital.

MAIN OUTCOMES AND MEASURES Ear samples were tested for pepsin A; cytokines interleukin (IL)-6, IL-8, and tumor necrosis factor; and bacterial culture inoculation. Data were analyzed by descriptive statistics and regression analysis to identify risk factors for the presence of pepsin A and to correlate pepsin A levels with cytokine levels, infection status, and clinical outcomes.

RESULTS Of the 129 patients, 199 earsamples were obtained; 82 samples (41%) and 64 patients (50%) were positive for pepsin A as measured by immunoassay. Pepsin A positivity correlated with age younger than 3.0 years (mean [SD], 2.3 [2.1] years in the positive group vs 3.3 [3.0] years in the negative group) and with all 3 cytokine levels (mean [SD] tumor necrosis factor, 29.5 [45.9] pg/mL in the positive group vs 13.2 [21.6] pg/mL in the negative group; IL-6, 6791.7 [9389.1] pg/mL in the positive group vs 2849.9 [4066.3] pg/mL in the negative group; and IL-8, 8282.2 [8122.3] pg/mL in the positive group vs 2925.1 [3364.5] pg/mL in the negative group [all P < .05]); however, logistic regression analysis showed that only IL-8 (odds ratio, 3.96; 95% CI, 1.3-12.0; P = .02) and age (odds ratio, 3.83; 95% CI, 1.1-12.7; P = .03) were significant independent variables. No statistically significant association was found with other parameters. Multiple linear regressions revealed that the levels of pepsin A were correlated with IL-8 levels (R² = 0.248; P < .001) and the need for second or third tubes 6 to 12 months after the first (R² = 0.102; P = .006). The presence of pepsin A in the middle ear was not associated with increased bacterial infection. Interleukin 8 was independent and significantly associated with both pepsin A levels and bacterial infection (R² = 0.144 and 0.263, respectively; P = .001 for both).

CONCLUSIONS AND RELEVANCE Extraesophageal reflux as indicated by the presence of pepsin A is closely involved in the middle ear inflammatory process and may worsen the disease in some children; however, a proof of cause and effect between extraesophageal reflux and middle ear inflammation requires further investigation.
Recent acute otitis media and otitis media with effusion are exceptionally common in the pediatric population. It has been suggested that extraesophageal reflux is one of the pathophysiologic mechanisms that appears to drive middle ear disease in children. Direct evidence of aspirating gastric contents into the middle ear was demonstrated by the detection of gastric pepsin A. Pepsin is produced by the chief cells of the stomach as the gastric proteolytic proenzyme pepsinogen, which has no enzymatic activity at a neutral pH level and is converted to active pepsin at a pH level lower than 4.0. At least 7 proteolytic pepsin isoforms are produced in the human stomach; pepsin A is the predominating isofrom and also the gastric specific form of pepsin. Pepsin and its inactive form pepsinogen in effusion samples of children with otitis media was reported to correlate with the number of pharyngeal reflux episodes, measured by pH monitoring. Our first study found that almost 15% of the middle ear samples from children with otitis media were positive for pepsin. Later, we evaluated a large cohort of patients with otitis media and a control group without otitis media. Pepsin was detected in the middle ear cleft of 20% of patients with otitis media undergoing tympanostomy tube placement, compared with 1.4% of controls. In addition, there was a significant association of the prevalence of pepsin in the middle ear with mucoid effusion, particularly purulent effusion, and younger age. Absence of gastric pepsin in control patients without otitis media provides compelling evidence that reflux plays a pathophysiologic role in the disease. A recent study also found that pepsin levels were significantly higher in children with otitis media compared with those without the disease.

One possible pathophysiologic mechanism of extraesophageal reflux disease (EERD)-related otitis media is eustachian tube dysfunction. This may be a result from a combination of factors, including inflammation of the mucosa from the corrosive property of gastric contents, particularly pepsin, acid, and bile acid; however, to our knowledge, the direct link between the presence of pepsin and inflammation in the middle ear has not been investigated. Our previous finding that purulent effusion and mucoid effusions were more likely to be positive for pepsin may suggest an association. This notion is further supported by previous studies showing that the concentrations of inflammatory mediators, such as interleukin (IL)-8, differ among subtypes of middle ear effusions. Different types of middle ear effusion may represent different degrees of middle ear inflammation or may reflect variable degrees of eustachian tube dysfunction. These findings support the belief that inflammation is intimately involved in the development of acute and chronic otitis media and that EERD might play a role in the inflammatory process.

The hallmark characteristic of otitis media is chronic inflammation that presents as the accumulation of a mucin-rich viscous effusion in the middle ear cleft. In addition, if infection occurs in the middle ear mucosa, the effusion undergoes a purulent phase. If eustachian tube dysfunction resolves during the acute episode, it is possible that the effusion may clear and the middle ear will become dry; however, when underlying causes are not identified, the resolution sometimes fails and the inflammation becomes chronic or recurrent otitis media. We hypothesized that the presence of pepsin A in the middle ear cleft might be responsible for or aggravate middle ear inflammation, thus preventing resolution of an acute episode. This might lead to increased middle ear infections and the need for multiple sets of myringotomy tubes. In this prospective study, to identify the potential risk posed by pepsin A in the middle ear inflammation process, we determined the presence of pepsin in middle ear aspirates and its correlation with inflammatory cytokines and clinical outcomes.

### Methods

#### Study Participants and Samples

After this study was approved by the institutional review board of Nemours Office of Human Subjects Protection, written informed consent was obtained from 373 patients who were scheduled for myringotomy with tube placement for otitis media. Boys and girls (age <18 years) who underwent myringotomy with tube placement for OME or recurrent acute otitis media were enrolled in the study (n = 373) regardless of other clinical conditions and medications. Only patients who presented with effusion (fluid) in their ears at the time of the procedure were sampled and included in the final study (n = 129). Patients who had dry ears at the time of sampling were excluded from the study. Demographic and clinical data including patient age, sex, body mass index (BMI), admitting diagnosis, operative procedure, and medical history of gastroesophageal reflux disease (GERD), allergy, and asthma were compiled from electronic medical records.

Samples were collected as described previously. Briefly, at the time of myringotomy (prior to placement of the tube), a suction cannula was placed through the myringotomy incision into the middle ear cleft. If fluid was present, 2 mL of sterile saline was flushed into the external auditory canal, allowed to flow into the middle ear cleft, and then aspirated with the suction cannula. If no effusion was present in the middle ear space, no sample was obtained and the patient was removed from the study. The fluid aspirated into the Lukens trap was transferred to a storage tube and stored at −20°C in preparation for assays. The operating surgeons categorized ears at the time of surgery as being “purulent,” “serous,” or “mucoid” based on the gross appearance of the aspirated material.

Prior to assays, the frozen samples were thawed on ice and gently inverted to mix. The samples were then centrifuged at 4°C to remove debris, and the supernatants were removed to use for protein, pH, pepsin A, and cytokine assays. The supernatants were initially assayed undiluted. However, when values were above the range of the upper limit of pepsin assay, the tests were repeated with diluted samples and the final results were corrected for the dilution factor.

Samples for routine bacterial culture were obtained with a sterile cotton swab immediately after collection and transferred to the microbiology laboratory for culture inoculation.

#### Pepsin A ELISA

A standard sandwich enzyme-linked immunosorbent assay (ELISA) method was applied to measure pepsin A concentra-
tion in the ear fluid. In brief, a 96-well microplate was coated with a monoclonal antibody custom made by Bio-Rad AbD Serotec against human pepsin A. After blocking with phosphate buffered saline–blocking buffer, 100-μL samples and porcine pepsin A standards (0–20 ng/mL) were applied to wells and incubated overnight at 4°C. The pepsin A captured on the wells was detected by incubating with biotin-conjugated polyclonal antibody to porcine pepsin A purchased from Abcam. The bound biotin-polyclonal antibody was detected by adding horseradish peroxidase (HRP)-streptavidin from Abcam. After biotin and streptavidin incubation, 3,3′,5,5′ tetramethylbenzidine substrate (Sigma-Aldrich) was added to react with HRP for 30 minutes, and 0.1% sodium fluoride solution was added to stop the reaction. The plate was read with a spectrometer at 650 nm. The amount of pepsin A in the samples was determined based on porcine pepsin A standard curves. The lower limit of sensitivity of the pepsin A ELISA assay is 0.1 ng/mL; pepsin A positivity is defined as pepsin A concentration higher than 0.25 ng/mL.

**Cytokine Assay**

All cytokines were measured using commercially available ELISA kits (Invitrogen). All measurements were performed according to the manufacturer’s instructions. In brief, standards and samples were placed in microplates precoated with specific capture antibodies. After the binding of antigens to the immobilized capture antibodies, a biotinylated second detection antibody was added and incubated for 1 hour. Streptavidin peroxidase was then added and followed by the addition of a substrate solution, which was acted on by the enzyme to produce color proportional to the concentration of cytokine present in the sample. The final concentration of a sample was determined based on the standard curve generated from the standards provided with the kit. Values below the lowest standard were classified as not detectable, and samples with values over the linear range were tested again with appropriate dilutions. Final concentration was calculated by correcting for the dilution factor. The cytokine values were expressed by nanograms per milliliter or picograms per milliliter of sample volume.

**Bacteria Culture**

Effusions were collected under sterile condition and transferred immediately to the microbiology laboratory for *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae* culture inoculation. Identification of bacteria grown on culture plates was carried out according to the standard bacteriological methods. A patient is categorized into the positive group if one of the culture assays has a positive result.

**Statistical Analysis**

Continuous variables were expressed as means (SD), and categorical variables as number (percentage) of patients. Data were initially analyzed with descriptive statistics to compare variables between the 2 groups. The comparison was assessed by t test for continuous variables and by χ² contingency analysis for categorical variables.

The logistic regression analysis was performed using SPSS statistical software system (version 19; IBM). Continuous variables with extreme skew and kurtosis such as cytokines, pepsin A, and age were transformed to the base-10 log (log₁₀) to achieve a normal distribution before regression modeling. Five patients had cytokine data missing. Because only 3.8% were missing, we used the mean imputation (substituting missing data with mean) method to handle missing data. In addition, we also repeated regression with the 5 cases deleted from the database (a simple omitting method), and the regression results were the same. In the final models, a P < .05 was used to indicate statistical significance. This analysis produced ORs that estimate in the multiplicative scale the risk factors associated with pepsin A positivity. Odds ratios (ORs) greater than 1 indicate increased risk, ORs lower than 1 indicated decreased risk, and ORs equal or close to 1 indicate no effect. Negative ORs indicated inverse correlation between the dependent and independent variables. Linear multiple regression was also performed to assess the effects of other independent variables on the levels of pepsin A.

**Results**

**Demographic Data and Pepsin Results**

A total of 373 patients who were diagnosed as having otitis media were initially enrolled in the study, but only 129 had effusion for sampling at the time of the procedure. Those who had dry ears were excluded from the study (n = 244). The final study group, comprising 72 boys and 59 girls, had a mean (SD) age of 2.8 (2.6) years, ranging from 7 months to 16 years old (Table 1). According to their medical records, less than 10% had clinical evidence of GERD, allergy, or asthma; 28% of them were using an allergy medicine; and less than 10% were using a proton pump inhibitor (Table 1).

| Table 1. Demographic Data of 129 Pediatric Patients Undergoing Myringotomy With Tube Placement for Otitis Media and Pepsin Results |
|-----------------|----------------------|
| **Patient and Sample Data** | **Value** |
| **Patients (n=129)** |  |
| Age, mean (SD), y | 2.8 (2.6) |
| Sex, No. |  |
| Male | 72 |
| Female | 57 |
| Clinical reflux, No. | 10 |
| Asthma, No. | 7 |
| Allergy, No. | 10 |
| On PPI therapy, No. | 10 |
| Adenotonsillar hypertrophy, No. | 6 |
| **Samples (n=199)** |  |
| Mucoid effusion, No. | 159 |
| Serous effusion, No. | 17 |
| Purulent effusion, No. | 23 |
| Positive patients, No. | 64 |
| Positive samples, No. | 82 |

Abbreviation: PPI, proton pump inhibitor.
A total of 199 ear samples were collected from the 129 patients; 70 had 2 ears sampled, and 59 had 1 ear sampled. Most samples (n = 159) were mucoid, 23 were purulent, and 17 were serous. On the basis of the defined cutoff value of 0.25 ng/mL of pepsin A, 82 samples from 64 patients were positive; 18 patients had both ears positive for pepsin A, and 46 patients had only 1 ear positive (Table 1).

Comparing Pepsin A–Positive and Negative Patients

Patients were divided into pepsin A–positive and pepsin A–negative groups based on whether pepsin A on the ELISA assay was above or below the established detectable value. For patients who had 2 ear samples, if one of the samples was positive, the patient was put into the pepsin A–positive group. The data from the negative ear of these patients were completely removed from the study and not used for further data analysis; only the data from the pepsin A–positive ear were used for these patients. Similarly, if both ear samples were positive, the data from the ear sample with a higher pepsin A value were used; the data from the other ear were removed and not used for further data analysis. Sixty-four patients had pepsin A above the established cutoff value in 1 or both ear samples, while 65 patients had either no pepsin A detected or had an amount below the established reference values in the ear samples (Table 2).

The demographic characteristics of the 2 groups are described in Table 2. Patients in the pepsin A–positive group were significantly younger than in the negative group. It was also noted that 52 children (81%) in the pepsin A–positive group were younger than 3 years, compared with 47 children (63%) in the pepsin A–negative group (P = .04). Furthermore, there were slightly more boys than girls in the positive group (62%) compared with the negative group (50%), but the difference was not statistically significant (P = .18). Body mass index in the 2 groups was comparable.

Samples were also tested for 3 cytokines (IL-6, IL-8, and tumor necrosis factor [TNF]) and bacterial cultures. All 3 cytokines were detected in the majority of samples from both groups, and the levels in the pepsin A–positive group were significantly higher than in the negative group (Table 2). Pepsin A–positive samples had a slightly higher percentage of positive bacterial cultures (36%) than the negative samples (21%), but the difference was not statistically significant (Table 2; P = .09). Furthermore, analyzing infection-positive cases (n = 26) revealed that neither the presence of pepsin A nor the levels of pepsin A were affected by the bacterial types (data not shown). Pepsin A–positive samples had a slightly higher percentage of purulent effusions than the negative samples, but the difference was not significant (P = .16). To control for disparity in sample collection, we also measured protein content, but no difference was found between the 2 groups (Table 2).

Analysis of clinical data revealed that patients who were positive for pepsin were more likely to require a second or third set of pressure-equalizing (PE) tubes 6 to 12 months after the initial tube placement (compared with the negative group; 28% vs 18%), but the difference was not statistically significant (Table 2). Furthermore, no difference was found in proton pump inhibitor and allergy medication use between groups (Table 2). Clinical evidence of reflux, allergy, and asthma were recorded, and few patients presented with any of these conditions (data not shown); therefore, no further analyses were performed on those data.

Regression Analysis

Logistic regression analysis was performed to determine potential predictors of pepsin A positivity. All variables that could not be
potentially contribute to or influence the detection of pepsin A were entered into the model. The results in Table 3 showed that only younger age and IL-8 were independently and significantly associated with pepsin A positivity when the influence from all other variables was controlled. The OR was 3.825 for age and 3.959 for IL-8 (Table 3). Children younger than 3 years were 3.825 times more likely than those 3 years or older to have pepsin A–positive results. With each 10-fold increase in IL-8 level, the chance of pepsin A positivity increased by 3.959 fold. Interleukin 6 and TNF were no longer associated with pepsin A positivity when other variables were controlled in the regression analysis (data not shown).

Multiple linear regressions were also performed to determine if the level of pepsin A was associated with other parameters when controlling for other variables. Stepwise regression showed that the only variables that were independently and significantly associated with pepsin A (log10) level were second tube placement and IL-8 levels (Table 4). None of the other factors, such as bacterial infection or effusion type, were correlated with the levels of pepsin A. The model explained 35% of the variance in pepsin A level, in which 24.8% correlated with the levels of pepsin A. The model explained 40.7% of the variance in IL-8 level, in which 26.3% correlated with the levels of IL-8. The model explained 10.2% of variability of IL-8, to which bacterial infection contributed significantly associated with pepsin A (log10) level were:

**Table 3. Logistic Regression Analysis of Pepsin A–Positive and Pepsin A–Negative Patients**

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>OR (95% CI)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;3.0 y</td>
<td>3.825 (1.2-12.7)</td>
<td>.03</td>
</tr>
<tr>
<td>Sex</td>
<td>2.364 (0.8-6.6)</td>
<td>.10</td>
</tr>
<tr>
<td>BMI</td>
<td>1.067 (0.9-1.3)</td>
<td>.54</td>
</tr>
<tr>
<td>PPI</td>
<td>3.978 (0.6-25.4)</td>
<td>.15</td>
</tr>
<tr>
<td>Allergy medication</td>
<td>1.493 (0.5-4.7)</td>
<td>.49</td>
</tr>
<tr>
<td>Second or third tube</td>
<td>1.720 (0.5-5.5)</td>
<td>.36</td>
</tr>
<tr>
<td>IL-8 (log10)</td>
<td>3.959 (1.3-12.0)</td>
<td>.02</td>
</tr>
<tr>
<td>Bacterial positive</td>
<td>1.090 (0.3-4.2)</td>
<td>.90</td>
</tr>
<tr>
<td>Purulent effusion</td>
<td>0.933 (0.2-3.9)</td>
<td>.92</td>
</tr>
<tr>
<td>Protein (log10)</td>
<td>0.499 (0.2-1.6)</td>
<td>.23</td>
</tr>
</tbody>
</table>

**Table 4. Multiple Linear Regression Analysis of Pepsin A and Interleukin (IL)-8 Levels**

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Coefficients*</th>
<th>R²</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8 (log10)</td>
<td>0.4490</td>
<td>0.248</td>
<td>.001</td>
</tr>
<tr>
<td>Second or third tube</td>
<td>0.3240</td>
<td>0.102</td>
<td>.006</td>
</tr>
<tr>
<td>R² for all variables</td>
<td></td>
<td>0.350</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial culture</td>
<td>0.4130</td>
<td>0.263</td>
<td>.001</td>
</tr>
<tr>
<td>Pepsin A (log10)</td>
<td>0.3920</td>
<td>0.144</td>
<td>.001</td>
</tr>
<tr>
<td>R² for all variables</td>
<td></td>
<td>0.407</td>
<td></td>
</tr>
</tbody>
</table>

* Standardized.

The age association with pepsin A has been demonstrated in our previous studies. In the present study, we further confirmed the correlation by performing regression analysis, which indicates that younger age had a unique contribution to pepsin A positivity. This may indicate that a more purulent eustachian tube in younger children allows access of refluxate into the middle ear cleft. It suggests that what was previously thought to be “physiologic reflux” might have both physiologic as well as pathophysiologic consequences because the levels of pepsin A were also correlated with the levels of IL-8. In susceptible children, reflux may be one of the causes or an aggravating factor of otitis media. Early studies revealed that middle ear levels of IL-6 and TNF were associated with age and might have different regulatory roles in the development stages of otitis media; however, we did not observe the age association with cytokine levels in the regression analysis when the contribution from other parameters, such as protein contents and effusion type, is entered into the model (data not shown). An alternative explanation is that the age range in our study population was not wide enough for us to see the difference. Studying a larger population with a broad age range will help to address the role of inflammatory cytokines in the development of otitis media in children.

In the present study, we did not observe correlation between effusion type and detection of pepsin A (levels or positivity). The percentage of purulent effusion was higher in the
pepsin A-positive group, but the difference was not statistically significant; although, in our previous study with a larger sample size that included dry and effusion ears, pepsin A prevalence was significantly higher in purulent and mucoid effusions than that in dry ears, and it was higher in purulent effusions than mucoid effusions.30,31 In the present study, we only included patients with effusions (wet ears); the majority had mucoid effusions (70%), and the rest were purulent (18%) or serous (13%). We believe that the lack of correlation in the present study was due to a relatively homogenous effusion type in the study population.

Although initial analysis showed a strong correlation between pepsin positivity and all 3 inflammatory cytokines, regression analysis demonstrated that the correlation with IL-6 and TNF was diminished when the influence of other variables, particularly bacterial infection, was controlled. Only IL-8 independently predicted pepsin A positivity and level, suggesting that IL-8 plays a role in pepsin-A-associated inflammation; the other 2 cytokines might play a lesser role. Animal experiments have revealed that exposure of the nasopharynx to simulated EERD leads to dysfunction of the eustachian tube and the development of inflammation demonstrated by increased goblet cell density, increased lymphocyte level, and polymorphonuclear leukocytes in the middle ear.35-37 Exposing cultured human airway epithelium to acid stimulates the adherence of Streptococcus pneumoniae to epithelial cells via nuclear factor–kappaB–mediated inflammatory response.38 Inflammatory mediators such as IL-6, IL-8, and TNF have been shown to be elevated in bronchoalveolar lavage of animals following acid aspiration.39 For the first time to our knowledge, we showed herein that the presence of pepsin A has a distinct association to the ongoing inflammation in the middle ears of children with otitis media.

Our results further confirm the notion that middle ear inflammation is multifactorial. In our present study, we have identified bacterial infection and pepsin A (gastric reflux) as the key factors in the ongoing inflammation. Elevation of IL-8 level is a marker for ongoing inflammation, which can be induced by bacterial infection and other injuries in the middle ear.40 Our study showed that both bacterial infection and the presence of pepsin A were correlated with elevated IL-8 level in the middle ear. Studies in vitro have shown that exposure to cytokines such as IL-8 in human middle ear epithelium can up-regulate mucin expression.41-42 In vivo, mucin messenger RNA expression in middle ear mucosa with mucoid otitis media was up-regulated 5- to 6-fold compared with controls without the condition.43-44 These studies suggest that elevation of IL-8 level and other cytokines in the middle ear plays an important role in the pathophysiologic mechanisms of otitis media by stimulating mucin overproduction, which in turn prevents normal mucociliary clearance and may be responsible for maintenance of the disease in the chronic stage.

On the basis of our current results and previous studies, the presence of pepsin A in the middle ear might have multifactorial consequence, including direct inflammatory injury of the mucosa from the corrosive property of gastric contents, particularly pepsin's proteolytic activity, coupled with the action of acid and bile acid. Furthermore, the inflammatory damage from gastric contents might induce overproduction of mucin in the middle ear via elevated cytokine (IL-8) level. In an immortalized middle ear line, Block et al20 recently showed that exposure of middle ear epithelium to acidic pH and/or pepsin increases Muc5b gene expression, which plays an important role in the pathophysiologic mechanisms of otitis media. They suggested that this perhaps explains how laryngopharyngeal reflux can contribute to otitis media by stimulating mucin production. Therefore, the presence of pepsin A in the middle ear, an indicator of a gastric reflux event, might have direct effects via acid and/or pepsin or via downstream mucin overproduction, in maintaining the disease in the chronic stage. This is supported by our finding that pepsin A levels were associated with second tube placement, an indication of ongoing and unresolved middle ear problems.

One essential question is whether middle ear pH needs to be maintained at acidic pH for pepsin to have its proteolytic effect on middle ear inflammation. It is well established that at neutral pH, pepsin has no enzymatic activity. The fact that pepsin A can be detected in a middle ear aspirate suggests that reflux episodes would have to be intense enough and/or frequent enough to cause the pepsin to be deposited in the middle ear space. It is most likely that the middle ear cleft would then have to be, at least periodically, exposed to an acidic pH, thus allowing the pepsin to be present. Our results suggest that such episodes were responsible for some of the inflammatory injury in the middle ear. With our current pepsin detection method, we cannot monitor the pH status and pepsin in the middle ear for a long period and can only provide a snapshot of these events. However, if medication with a histamine type 2 blocker or proton pump inhibitor consistently maintains stomach pH near neutral, the chance of detection of pepsin would be reduced; thus, the effects of pepsin on the middle ear inflammation would be diminished.

It has been shown previously that the presence of bacteria was associated with increased inflammation and elevated cytokine levels in the middle ear of children with recurrent and/or chronic otitis media.40 Similarly, in animal studies, directly injecting a very small number of viable pneumococci into the middle ear resulted in elevated levels of IL-6, IL-8, and TNF in the middle ears.40-43 As expected, we also found a strong correlation between bacterial infection and all 3 inflammatory cytokines, confirming the role played by bacterial infection on the level of inflammation in the ears and its independent contribution to middle ear inflammation (data not shown). However, positive pepsin or increased pepsin A levels did not show as strong an association with infection as we had initially hypothesized. It is possible that the effect of gastric reflux on the middle ear is preferentially linked with other types of bacteria in the middle ear. However, perhaps due to the small sample size (26 bacterial positive), no correlation was revealed between the 3 types of bacteria and pepsin A in our study.

An interesting finding of this study was that the level of pepsin A in the ears independently predicted whether a second or third tube placement is needed 6 to 12 months after the initial tube placement. This correlation between the presence of pepsin A and clinical outcome may suggest that EERD plays a role in the persistence of otitis media and further sup-
ports the concept that gastric reflux in younger children may be pathophysiologic. A limitation of the study is that we did not collect data on the indications for having a second or third tube placement (eg, recurrent acute otitis media vs chronic middle ear effusions). It has been reported that younger age at the initial tube placement is associated with a higher incidence of additional tube placement, while tympanostomy tube with adenoidectomy reduces the probability to have a second bilateral myringotomy and tubes (BMTs); however, we did not find a correlation between age and the need for a second or third tube placement in our study participants. Investigating children with post-bilateral myringotomy and tube otorrhea will help to further delineate the role of EERD in otitis media.

Most of the children in our study were not confirmed to have GERD by objective tests such as 24-hour pH impedance ondbilateral myringotomy and tubes (BMTs); therefore, we did not find a correlation between age and the need for a second or third tube placement in our study participants. Investigating children with post-bilateral myringotomy and tube otorrhea will help to further delineate the role of EERD in otitis media.

Conclusions

The question of whether GERD is a prerequisite for the development of extraesophageal complications is being continuously debated. Herein, we revealed that regardless of the status of GERD, extraesophageal reflux as indicated by the presence of pepsin A is closely involved in the inflammatory process of middle ear.

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