Nasopharyngeal Biofilm-Producing Otopathogens in Children with Nonsevere Recurrent Acute Otitis Media

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Abstract

Objective. Bacterial biofilms have been detected in biopsies of the adenoid and middle ear mucosa of otitis-prone children and children with chronic middle ear media. However, the invasiveness of biopsy makes it unsuitable for routine clinical practice, especially in pediatrics. This study aimed to investigate nasopharyngeal biofilm-producing otopathogens (BPOs) of nasopharyngeal swabs (NPS) in children with a history of nonsevere recurrent acute otitis media (RAOM) and healthy controls.

Study Design. A cross-sectional study with planned data collection.

Setting. University of Milan.

Subjects and Methods. Transoral NPS were taken from infants and children aged 10 months to 11 years with nonsevere RAOM or healthy controls without adenoid hypertrophy. Nasopharyngeal colonization by otopathogens was assessed by means of microbiological cultures and standard bacterial identification, as well as nasopharyngeal BPOs by means of spectrophotometric analysis.

Results. The study involved 113 children (56.6% males; median age 40 months; range, 10-132 months): 58 with a history of nonsevere RAOM (51.3%) and 55 controls (48.7%). Otopathogens were significantly more frequently detected in the RAOM group (24/58, 41.4%) than in controls (8/55, 14.5%; $P = .003$); the main pathogens were respectively Haemophilus influenzae (12/24, 50.0%) and Streptococcus pyogenes (3/8, 37.5%). Nasopharyngeal BPOs were more frequently isolated in the RAOM group (17/58, 29.3%) than in controls (6/55, 10.9%; $P = .02$). H. influenzae (12/17, 70.6%) was confirmed as the main pathogen in the RAOM group.

Conclusion. The presence of nasopharyngeal BPOs is an important factor favoring RAOM; it is therefore useful investigating biofilms even in children with nonsevere recurrences of AOM without adenoid hypertrophy.

Keywords
biofilm, otopathogens, recurrent acute otitis media, nasopharynx

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Bacterial biofilms are involved in the chronicity of infections and resistance to antibiotic treatments, thus having a considerable negative impact on patients’ quality of life and a significant effect on public health.1,2 It has been shown that they play a role in upper respiratory tract diseases, including acute and chronic middle ear diseases,3,4 and it has been suggested that the formation of biofilms by pneumococci is a potential cause of treatment failure in the chinchilla middle ear model.5 The nasopharynx and surrounding tissues of children act as important reservoirs of resistant bacterial biofilms, which have been detected in biopsies taken from adenoid and/or middle ear mucosa of children with recurrent infections,6-8 chronic middle ear effusion (OME),9,10 and chronic suppurative otitis media (CSOM).11 Scanning electron microscopy has shown that biofilms cover almost all of the mucosal surface of adenoids removed from children with recurrent acute otitis media (RAOM).12,13

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The invasiveness of the biotic detection of biofilms makes it unsuitable in routine clinical practice, especially in children. We have previously found that the diagnostic accuracy of nasopharyngeal swabs (NPS) in detecting biofilm-producing bacteria in a cohort of children with chronic adenoiditis is generally poorer than that of adenoidal biopsies. However, given the fairly good sensitivity and positive predictive value of NPS and the unsubstantial difference in pathogen identification, the use of such an economic and simple means of detecting biofilm-producing otopathogens (BPOs) may be useful, especially in positive cases. In addition, it has been shown that children with RAOM are more frequently colonized by nasopharyngeal otopathogens than controls and that the bacterial colonization patterns vary with the type and severity of otitis media.

The aim of this study was to investigate the feasibility of using spectrophotometric analysis of NPS to quantify biofilm formation in children with a history of nonsevere RAOM and healthy controls. Spectophotometric assessment was chosen as a simpler and cost-effective quantitative method compared with expensive and extensive sample preparation needing electron microscopic techniques.

**Methods**

**Study Design and Setting**

This cross-sectional study with planned data collection, which was approved by the Ethics Committee of the University of Milan, was carried out between October 2009 and October 2010 in the Department of Maternal and Pediatric Sciences and the Department of Specialist Surgical Sciences of Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico (recruitment, specimen collection, and statistical analysis) and at the Clinical Microbiology Laboratory of the University of Milan’s Department of Preclinical Science (DISP) LITA (microbiological assessment). Written informed consent was obtained from the children’s parents or legal guardians, and the older children were also asked for their assent.

**Study Population**

The study involved children aged 10 months to 11 years with a history of nonsevere RAOM (defined as 3 episodes in the preceding 6 months, with the most recent episode occurring at least 4 weeks before and no more than 4 episodes in the preceding 12 months). The episodes of acute otitis media (AOM) were documented by medical records and had to include any combination of fever, earache, irritability, and hyperemia or opacity accompanied by bulging of the tympanic membrane or otorrhea. At least 2 episodes had to be supported by otoscopy and tympanometric findings. At the time of sampling, the children had to be free of AOM and middle ear effusion. The exclusion criteria were acute febrile illness; upper respiratory tract infection or antibiotic therapy in the previous 14 days; concomitant systemic diseases; craniofacial, neuromuscular, immunological, syndromic, or defined genetic abnormalities; and previous ear surgery or adenoidectomy. Age-matched children without any evidence or history of AOM were used as controls; these children were outpatients or inpatients undergoing minor surgery.

**Study Procedures**

At the enrollment visit, a complete clinical history was taken, and the children underwent a detailed clinical examination, with particular attention given to the ears, nose, and throat using pneumatic otoscopy (Model 20200; Welch Allyn, Skaneateles Falls, New York) and tympanometry (Amplaid 770; Amplifon, Plymouth, Minnesota). Obstructive hypertrophic adenoids (Cassano grade 3 or more) were detected by means of nasal fiber-optic endoscopy. The following variables were recorded: sex, age, breastfeeding, day care attendance, the presence of older siblings, passive smoking exposure, allergy (documented by a positive skin prick test within the previous 12 months), the use of a pacifier, the time since the last AOM episode and antibiotic consumption, any history of spontaneous eardrum perforation with otorrhea, the season of assessment, and the previous administration of conjugate *Haemophilus influenzae* type b (Hib) and/or pneumococcal vaccine.

When AOM was diagnosed, amoxicillin (80 mg/kg/d) plus clavulanic acid (ratio 7:1) was given for 10 days, and NPS collection was postponed until its complete clinical resolution. No other treatments were allowed during the follow-up period.

**Specimen Collection**

All of the transoral NPS were taken by the same specially trained examiner (PM) using an extra-thin flexible wire swab (Mini-Culturette; Becton Dickinson, Cockeysville, Maryland) with a 30° bent tip, which was inserted through the mouth and positioned 1 to 1.5 inches into the nasopharynx (taking care not to touch the uvula or tongue) and left in place for 4 to 6 seconds.

**Bacterial Growth and Identification**

A nasopharyngeal culture was obtained to determine colonization with *Streptococcus pneumoniae*, *H influenzae*, *Moraxella catarrhalis*, or *Streptococcus pyogenes*. All of the specimens were inoculated into Stuart transport medium tubes (Copan Venturi Transysystem, Brescia, Italy) and processed within 2 hours by the clinical microbiology laboratory. The swabs were transferred to tubes containing 1 mL of brain/heart infusion broth (Difco, Detroit, Michigan) with the addition of 5% of sheep’s blood and vortexed for 30 seconds. The samples were appropriately diluted in the same medium, and 100 μL of each dilution was inoculated into horse blood, chocolate, and Columbia CNA agar plates. All of the plates were incubated in a 10% enriched carbon dioxide atmosphere for 18 to 24 hours at 37°C, and their morphological and gram stain characteristics were microscopically observed. *S pneumoniae*, *S pyogenes*, *H influenza*, and *M catarrhalis* were isolated and identified using the standard laboratory procedures of latex agglutination (Oxoid, Basingstoke, UK) and API System (bioMérieux, Marcy L’Étoile, France).
Evaluation of Biofilm Formation

Biofilm formation was evaluated spectrophotometrically using the method by Christensen et al.16 S pneumoniae, S pyogenes, and M catarrhalis were inoculated in 10 mL of brain-heart infusion (BHI) containing 5% sheep’s blood; H influenzae was inoculated in 10 mL of Haemophilus test medium (HTM) and incubated for 18 hours at 37°C in 10% CO2. Aliquots of 20 μL of broth culture were then pipetted into 3 wells in 96-well plates (BD Falcon, Franklin Lakes, New York) containing 180 μL triptone broth solution (TSB) with the addition of 5% sheep’s blood for S pneumoniae, S pyogenes, and M catarrhalis or HTM for H influenzae. After incubation for 18 hours as above, the nonadherent bacteria were removed, and the wells were rinsed 3 times with 200 μL of sterile physiological solution and stained with 200 μL of crystal violet; the excess stain was removed 15 minutes later, and each well was rinsed 3 times with distilled water.

The amount of the obtained biofilm was determined by means of spectrophotometry (wavelength 595 nm) using a microplate reader (Biorad, model 680; Segrate, Milan, Italy).

On the basis of the difference between the optical density (OD) of the biofilm bacteria and that of the negative control (ODb), the bacteria were graded as nonproducers (OD ≤ ODb), weak producers (ODb < OD ≤ 2 × ODb), moderate producers (2 × ODb < OD ≤ 4 × ODb), or strong producers (4 × ODb < OD).

Statistical Analysis

The prevalence and distribution pattern of nasopharyngeal otopathogens and nasopharyngeal BPOs were assessed. Multivariate logistic regression analysis was used to determine whether the presence of nasopharyngeal BPOs was associated with the study group, sex, age, breastfeeding, day care attendance, presence of older siblings, passive smoking exposure, allergy (documented by a positive skin prick test within the previous 12 months), regular use of a pacifier, the presence of older siblings, passive smoking exposure, allergy, previous vaccine, upper respiratory tract infections in children (ie, day care attendance, the use of pacifier, the presence of older siblings, passive smoking exposure, allergy, previous vaccine, and season) and the variables related to the severity and clinical characteristics.

Results

Study Population

The study involved 113 children (56.6% boys; median age 40 months; range, 10-132 months). As about 75% of children were younger than 5 years and as a preliminary analysis failed to find any significant difference between children ≤5 and >5 years, we decided to perform the final analysis on the expanded age range, including 58 children (51.3%) with a history of nonsene RAOM and 55 controls (48.7%).

About one-third of the children had experienced previous spontaneous otorrhea; none had obstructive hypertrophic adenoids. Table 1 summarizes their main demographic and clinical characteristics.

Microbiological Findings

Table 2 shows all of the main microbiological results. Otopathogens were significantly more frequently detected in the children with a history of RAOM (24/58, 41.4%) than in the controls (8/55, 14.5%; P = .003); the main pathogens were respectively H influenzae (12/24, 50.0%) and S pyogenes (3/8, 37.5%). The nasopharyngeal carriage rates did not depend on a history of spontaneous otorrhea. The distribution of otopathogens was not significantly different in the 2 study groups. Nasopharyngeal BPOs were more frequently isolated in the RAOM group (17/58, 29.3%) than in the controls (6/55, 10.9%; P = .02), and H influenzae (12/17, 70.6%) was confirmed as the main pathogen in the RAOM group. There was no between-group difference in the type of otopathogens, except for H influenzae (P = .005), or their ability to produce biofilms. None of the otopathogens was a strong biofilm producer, but 3 (M catarrhalis [n = 2]; S pneumoniae [n = 1]) collected from the RAOM group and 2 (S pneumoniae) from the control group were moderate biofilm producers. The prevalence of biofilm-producing otopathogens did not depend on a history of spontaneous otorrhea.

Epidemiological Variables

Being a child with a history of RAOM was the main predictor of the presence of nasopharyngeal BPOs (odds ratio [OR] = 4.69, SE = 2.681, P = .007). The $r^2$ was 17.2%, suggesting a weak fit between the model and the data.

None of the other epidemiological variables was associated with the presence of nasopharyngeal BPOs. In particular, the variables known to modify the natural history of upper respiratory tract infections in children (ie, day care attendance, the use of pacifier, the presence of older siblings, passive smoking exposure, allergy, previous vaccine, and season) and the variables related to the severity and temporal sequence of the disease (ie, previous spontaneous otorrhea, the time since the last AOM episode, and the last antibiotic course) were not statistically related to the presence of nasopharyngeal BPOs.

Discussion

Our data suggest further evidence about the relationship between nasopharyngeal bacterial biofilms and recurrent middle ear diseases in children. In addition, this is the first study using a nonaggressive sampling procedure to evaluate the presence of nasopharyngeal biofilms in pediatric outpatients.
Table 1. Demographic and Clinical Characteristics of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>RAOM (n = 58)</th>
<th>Controls (n = 55)</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boys</td>
<td>35 (60.4)</td>
<td>29 (52.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Median age, mo (range)</td>
<td>39 (15-115)</td>
<td>40 (10-132)</td>
<td>NS*</td>
</tr>
<tr>
<td>Breastfeeding</td>
<td>44 (75.9)</td>
<td>46 (83.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Full-time daycare attendancec</td>
<td>39 (67.3)</td>
<td>39 (70.9)</td>
<td>NS</td>
</tr>
<tr>
<td>At least 1 older sibling</td>
<td>23 (39.6)</td>
<td>28 (50.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Exposure to passive smoking</td>
<td>18 (31.0)</td>
<td>15 (27.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Allergy</td>
<td>11 (19.0)</td>
<td>10 (18.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Regular use of pacifier</td>
<td>19 (32.7)</td>
<td>6 (10.9)</td>
<td>.022</td>
</tr>
<tr>
<td>Time since previous episode of AOM, mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9 (15.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24 (41.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥3</td>
<td>25 (43.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time since previous antibiotic course, mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10 (17.3)</td>
<td>7 (12.7)</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>27 (46.5)</td>
<td>26 (47.3)</td>
<td></td>
</tr>
<tr>
<td>≥3</td>
<td>21 (36.2)</td>
<td>22 (40.0)</td>
<td></td>
</tr>
<tr>
<td>At least 2 previous episodes of spontaneous otorrhea</td>
<td>21 (36.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous Haemophilus influenza type b vaccine</td>
<td>58 (100)</td>
<td>55 (100)</td>
<td>NS</td>
</tr>
<tr>
<td>Previous pneumococcal vaccine</td>
<td>30 (51.7)</td>
<td>21 (38.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Obstructive hypertrophic adenoids</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>September-November</td>
<td>9 (15.5)</td>
<td>8 (14.6)</td>
<td>NS</td>
</tr>
<tr>
<td>December-February</td>
<td>26 (44.8)</td>
<td>22 (40.0)</td>
<td></td>
</tr>
<tr>
<td>March-May</td>
<td>11 (19.0)</td>
<td>13 (23.6)</td>
<td></td>
</tr>
<tr>
<td>June-August</td>
<td>12 (20.7)</td>
<td>12 (21.8)</td>
<td></td>
</tr>
</tbody>
</table>

Values presented as No. (%) unless otherwise stated. Abbreviations: AOM, acute otitis media; NS, not significant; RAOM, recurrent acute otitis media; —, not applicable.

aFisher exact test (unless otherwise stated).
bAnalysis of variance (ANOVA).
cFive days/wk, 6 to 8 h/d.

Table 2. Nasopharyngeal Microbiological Characteristics of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>RAOM (n = 58)</th>
<th>Controls (n = 55)</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carriers of otopathogens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moraxella catarrhals</td>
<td>7 (29.2)</td>
<td>2 (25.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>3 (12.5)</td>
<td>2 (25.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>2 (8.3)</td>
<td>3 (37.5)</td>
<td>.085</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>12 (50.0)</td>
<td>1 (12.50)</td>
<td>NS</td>
</tr>
<tr>
<td>Carriers of BPOsb</td>
<td>17 (29.3)</td>
<td>6 (10.9)</td>
<td>.019</td>
</tr>
<tr>
<td>M catarrhals</td>
<td>1 (5.9)</td>
<td>2 (33.4)</td>
<td>NS</td>
</tr>
<tr>
<td>S pneumoniae</td>
<td>3 (17.6)</td>
<td>2 (33.3)</td>
<td>NS</td>
</tr>
<tr>
<td>S pyogenes</td>
<td>1 (5.9)</td>
<td>2 (33.3)</td>
<td>NS</td>
</tr>
<tr>
<td>H influenzae</td>
<td>12 (70.6)</td>
<td>0 (0.0)</td>
<td>.005</td>
</tr>
<tr>
<td>Biofilm production grading</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>14 (82.4)</td>
<td>4 (66.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Moderate</td>
<td>3 (17.6)</td>
<td>2 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BPO, biofilm-producing otopathogens; NS, not significant; RAOM, recurrent acute otitis media.

aFisher exact test.
bThree patients in the RAOM group carried 2 different BPOs.
affected by nonsevere RAOM and not undergoing surgery. Moreover, no direct comparison of children with RAOM with or without otorrhea has ever been made before.

The finding that nasopharyngeal colonization by otopathogens is significantly more prevalent in children with a history of RAOM than in controls is in line with those of previous studies showing increased carriage of *H influenzae* in otitis-prone children,20 and corroborates the findings of our previous study showing a higher rate of otopathogens carriage in children with RAOM than in controls.15

The greater prevalence of nasopharyngeal BPOs in children with a history of RAOM than in controls is in line with the findings of previous animal21 and human studies.9,22 It is also in line with those of Zuliani et al,13 who showed that biofilms were significantly more prevalent in the adenoids of children with RAOM, although their children had a history of severe RAOM (4 episodes in 6 months or 6 in 12 months) that made them eligible for surgery.

The proportion of children harboring nasopharyngeal BPOs in our population was lower than observed in other studies,11,12 probably because of the different sampling techniques (ie, nasopharyngeal swab vs mucosal biopsy) and the different microbiological methods (ie, spectrophotometry vs scanning electron microscopy). In particular, the poor negative predictive value of NPS in detecting biofilm-producing bacteria, which is probably due to the well-known resistance of biofilm to mechanical injuries such as swab rubbing, needs to be taken into account as a possible explanation of false-negative results as shown in our previous study.14

*H influenzae* was identified as the main pathogen involved in biofilm formation in the group of children with RAOM, thus confirming previous observations,10,22,23 about the relationship between *H influenzae* biofilm producer and recurrent middle ear infection. It is worth noting that all of the isolates of *H influenzae* were biofilm producers, a finding that is in line with those of Moriyama et al,22 who found that about 84% of the clinical isolates of *H influenzae* were biofilm-forming strains. In our data, *S pneumoniae* biofilm producers were not strongly associated with RAOM, as they were detected in only about 18% of otitis-prone children, without any significant difference in detection rates between clinical groups. This finding is in line with those of Nistico et al,10 and we can speculate that it may be partially attributable to the fact that a substantial number of the children in our case series had previously received anti-pneumococcal vaccine.

Most of the BPOs were weak biofilm producers. This conflicts with the findings of Camilli et al,24 who reported strong biofilm production in 64.4% of their isolates of *S pneumoniae*. One possible explanation is that they evaluated isolates from patients with invasive diseases, whereas ours were nasopharyngeal colonizing isolates from children with a history of disease; in addition, one may argue that nonaggressive NPS might be responsible for an easier recruitment of superficial bacteria, compared with less mature and aggressive biofilm producers hidden within the depth of biofilm.

None of our children had obstructive hypertrophic adenoids, and the nasopharyngeal colonization pattern and/or presence of biofilm was not related to the presence or otherwise of adenoids. Our data corroborate the findings of our previous study and suggest that otopathogen biofilms seem to play a role per se and are not necessarily mediated by the presence of adenoids.15

None of the epidemiological variables assessed (other than being a child with nonsevere RAOM) seemed to play a role in determining the presence of nasopharyngeal BPOs; in particular, despite a greater prevalence of regular users of pacifiers among RAOM children, as documented in our previous study,15 we failed to find any association between this habit and the presence of nasopharyngeal BPOs.

The absence of an association between the presence of nasopharyngeal BPOs and episodes of acute otorrhea with spontaneous eardrum perforation has never been reported before. There are a number of possible explanations associated with this finding, including that biofilm presence is not associated with the tympanic membrane perforation complication of AOM. However, further studies should be conducted as to the association of biofilms and severe AOM, including studies examining the biofilm presence at the time of the complication.

The finding of nasopharyngeal BPOs in a limited number of children without middle ear infections (10.9%) has been previously described by other authors10 and may be related to the colonization of the spare adenoidal tissue by opportunistic biofilm-producing bacteria that do not trigger any infectious exacerbation.

This study has some limitations. No attempt was made to ascertain the serotypes or the resistance of *S pneumoniae* isolates as this was considered beyond the scope of the study. However, these data would not have added relevance to our results, as no association has been found between the ability to form biofilm and serotypes, antibiotic resistance, or clinical presentation.24,25 Moreover, no attempt was made to distinguish Hib from non-typeable *H influenzae*. However, it is conceivable that the role of Hib would have been negligible as all of our patients had received anti-Hib vaccine, which has been recommended for all 3-month-old infants in Italy since 2002.

We conclude that the presence of nasopharyngeal BPOs should be considered and investigated even in children with nonsevere RAOM who do not need adenoid surgery. Clinicians should be encouraged to avoid antibiotics that are ineffective and may further select resistant organisms. In addition, while awaiting clinically suitable and efficacious treatment for nasopharyngeal biofilms, it is desirable to test new disposable devices aimed at reaching anatomic recesses where biofilm can nestle, such as the nasopharynx and peri-tubricar area.

Finally, the detection of nasopharyngeal BPOs in children during the interval between episodes of AOM suggests that bacteria are not removed from the nasopharynx after an acute episode. This raises some doubts about the adequacy of the concept of AOM “recurrence” rather than AOM...
chronicity and suggests the need for a new classification of acute, recurrent, and chronic infections.

Author Contributions

Sara Torretta, data analysis and interpretation, drafting and final approval; Paola Marchisio, conception and design, data analysis and interpretation, drafting and final approval; Lorenzo Drago, acquisition of data, drafting and final approval; Elena Baggi, acquisition of data, drafting and final approval; Elena De Vecchi, acquisition of data, drafting and final approval; Werner Garavello, data interpretation, revising the article, final approval; Erica Nazzari, acquisition of data, drafting and final approval; Paola Marchisio, data interpretation, revising the article, final approval; Sara Torretta, data analysis and interpretation, drafting and final approval; Susanna Esposito, conception and design, data analysis and interpretation, revising the article, final approval.

Disclosures

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