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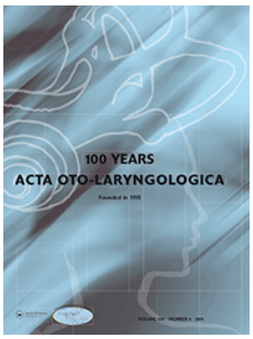

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RESEARCH ARTICLE

Bacterial biofilm in adenoids of children with chronic otitis media. Part I: a case control study of prevalence of biofilms in adenoids, risk factors and middle ear biofilms

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ABSTRACT

Background: Biofilms are communities of bacteria embedded in a self-produced glycocalyx matrix. Adenoids have been shown to harbor bacterial biofilms.

Aim/objectives: To compare the prevalence of biofilms in adenoid of children with chronic otitis media (COM) (group1) versus a control group without any COM (group 2) having adenoids removed because of hypertrophy.

Material and methods: One hundred and three children were prospectively enrolled in this case-control study, group 1 ($n = 52$) and group 2 ($n = 51$). The main outcome measurement was the prevalence of biofilm in adenoidectomy specimens analyzed using confocal laser scanning microscopy. Children in group 1 who had middle ear (ME) effusion and requiring the insertion of a tympanostomy tube underwent biopsy of the ME mucosa and effusion sampling.

Results: Biofilms were found in adenoids' specimens of both groups and in the ME biopsy and effusion. The biofilm prevalence in adenoids was 63.5% (33/52) in group 1 and 47.1% (24/51) in group 2. Day nursery and previous antibiotics intake were significantly more frequent in group 1 than in group 2.

Conclusions and significance: This case-control study demonstrates that adenoid tissue in children with COM contains more mucosal biofilms than adenoid tissue removed for hypertrophy. Biofilm was seen in ME biopsies and effusion.

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Introduction

Bacterial biofilms are defined as communities of bacteria embedded in a self-produced glycocalyx matrix [1,2]. In the beginning, free-swimming planktonic bacteria attach to a surface and then bacteria develop a biofilm by initiating a cell to cell communication known as quorum-sensing building up a matrix of polysaccharides encasing various communities of bacteria. This matrix protects bacteria and offers them different properties like living in a dormant state, resistance to both host immune defenses and antibiotics [3,4]. According to local conditions, some parts of the biofilm may detach and colonize new niches. Therefore, biofilm formation and development is a concept of persistence of bacteria in the human body and provides an understanding of some chronic and/or recurrent infections [3,4].

Adenoids were proven to harbor biofilms [5]. Clear visualization of the biofilms needs to show bacterial communities and the matrix [6]. The demonstration of bacterial biofilms in adenoids human tissues by showing both the bacterial cells and the surrounding matrix has been provided using confocal laser scanning microscopy (CLSM) with double fluorescent staining technique [6]. Since there is evidence of a bacterial load in adenoid tissue, it is supposed that adenoids play a central role in the physiopathology of chronic otitis media (COM) [5,7]. COM is a very common disease in children. The presence of bacterial biofilms on the adenoids suggests that biofilms may be responsible for COM with effusion. Biofilms associated with adenoids may act as a reservoir for the persistence of oto-pathogens in the nasopharynx that can give rise to COM [7].

In an effort to relate the findings of bacterial biofilm with the clinical presentation, the present prospective, unrandomized case-control study was designed in order to compare the prevalence of biofilms in adenoid tissues in two populations of children. In group 1 (cases), children with COM had adenoids removed because of COM whereas,

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in group 2 (controls), children without COM had their adenoids removed because of hypertrophy. In addition, some children with COM (cases group 1) had also ventilation tube insertion and effusion sampling. Biofilms were searched in middle ear (ME) biopsies and ME effusion.

Materials and methods

Study design

This is a prospective monocentric unrandomized case-control study comparing the prevalence of biofilms between 2 groups of children matched for age and gender. Cases (Group 1) were children who underwent adenoidectomy for COM. Controls (Group 2) were children who underwent adenoidectomy for obstructive adenoids without COM. In group 1, when children with COM needed to have a ventilation tube inserted then a biopsy of ME mucosa and effusion liquid were collected. The specimens were prepared for confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). The main outcome measurement was the prevalence of mucosal biofilm formations in adenoidectomy specimens analyzed using CLSM with double staining to visualize both the bacteria and the glycocalyx matrix. The following clinical features were recorded for searching risk factors: age, sex, breastfeeding, passive smoking, day nursery, young siblings, anemia, acid reflux, allergy, number of previous antibiotics treatments. Institutional review board approval was obtained. Informed consent was obtained for each child.

Inclusion/exclusion criteria

Children must be between 18 months and 10-year-old of age. In group 1 (cases of children with COM) children had OME for more than 3 months as diagnosed by an ENT physician. In group 2, (control group of children without COM) children had adenoid hypertrophy confirmed by nasal endoscopy without any other ENT infection and with normal tympanic membrane. Exclusion criteria were: absence of parents' consent, age under 18 months or over 10 years, children with Down syndrome or deformity of the soft palate, children with immunosuppression or HIV positive status, children with another ENT infection or with suspicion of tumor.

Main outcome measurement: prevalence of biofilms in adenoids with CLSM

Immediately after adenoidectomy, each specimen was aliquoted into snap-frozen in cold isopentane on dry ice and stored at −80 °C. One part of the adenoids was dedicated to CSLM to compare the prevalence of biofilms in the two groups of children, another part to SEM in order to visualize biofilms on the surface of the specimens. In group 1 of children with COM, when a ME biopsy and effusion sampling were obtained, the specimens were prepared and studied with CLSM.

For CLSM, the frozen specimens were cut to a thickness of 10 µm at −24 °C using cryostat CM 3050S (Leica Microsystems, Bensheim, Germany) and fixated in 70% acetone. The obtained sections were then processed for double staining. They were washed three times with PBS, and first stained with propidium iodide 15 µmol/L for 5 minutes at room temperature to detect bacterial cells in red. After washing with PBS, the sections were incubated with 50 µg/mL Concanavalin A fluorescein isocyanate-conjugated (ConA-FITC C7642; Sigma-Aldrich Inc, St Louis, Mo) for 5 minutes at room temperature to stain the glycocalyx in green. The sections were then successively washed in PBS and demineralized water and embedded in Gelvatol/DABCO. The sections were examined using an Axioplan upright microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Biorad MRC1024ES scan head (Hercules, CA) with krypton/argon laser for visualization of ConA-FITC (number of signals acquired, 488 nm; emission 552 disconnecting filter (DF) 32 nm) and propidium iodide (number of signals acquired, 568 nm; emission 605 DF, 32 nm). Digital images of the CLSM optical section were collected using the Lasersharp 2000 software (Biorad, Hercules, CA). Merged red and green images were obtained into single TIFF format and converted to high-quality Joint Photographic Experts Group (JPEG) files using Adobe Photoshop 7.0 software (Adobe Systems Inc, San José, CA).

SEM was used in order to search for biofilms on the surface of the specimens. The specimens were washed in phosphate buffered solution (PBS), fixed with 1.5% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer pH 7.4 for 24 hours at 4 °C on a rotary shaker. The samples were ten subsequently dehydrated through a graded series of acetone solutions (70%, 80%, 90%, 96% and 100% acetone) for 20 minutes at room temperature, and the critical point drying was performed. The specimen was then orientated, mounted on metal stubs, and sputter coated with gold using a Polaron 5000 Sputtering System (Watford, England) prior to imaging. The specimens were examined in a JSM6400 scanning electron microscope (JEOL, Tokyo, Japan) with digital imaging capabilities. The images were collected at an acceleration voltage of ∼5.0 kV, a filament current of ∼10–10 A, a working distance of ∼15 mm. All images were digitized as high-resolution Tagged Image File Format (TIFF) computer files (resolution 635 dpi) and then converted to high-quality JPEG files using Adobe Photoshop 7.0 software (Adobe System Inc, San Jose, CA).

Image analysis

Three investigators (PV, GL, and RK) evaluated the images independently in a blinded retrospective manner according to the criteria previously published to retain a specimen as containing a bacterial biofilm [6]. Briefly, criteria were: (1) presence of bacteria recognized by size, morphology and for CLSM by red fluorescent propidium iodide staining; (2) presence of glycocalyx shown on CLSM images by bright green fluorescence due to ConA-FITC staining; (3) more than one biofilm identified; (4) the absence of artifacts of

dehydration or cutting for SEM and CLSM respectively; and (5) the absence of exclusion criteria such as bacteria located outside the specimen that could account for potential infection during section preparation.

Statistical analysis

The demographic and medical features between the two groups were compared and analyzed using a student test. For the main outcome measurement (prevalence of biofilms in adenoids' tissues), the hypothesis of a difference of biofilm prevalence between the cases (group 1 children with COM) and the control group (children without COM) was tested by a conditional logistic regression model. The number of patients to include in order to reach a sufficient statistical power (80%) according to Chi2 test was 104, i.e. 52 patients per group. Statistical significance was set at $<.05$.

Results

One hundred and four children were enrolled in this case-control study. One child had to be excluded from the study in the absence of one of the two parent consent. Therefore, a total of 103 children was included as group 1 ($n = 52$) children with COM and group 2 ($n = 51$) children without COM as a control group.

Adenoid tissues

Figure 1 shows representative images of bacterial biofilms that were found in adenoids' specimens in both groups of children with (cases, group 1) or without (controls, group 2) COM. Biofilms were found sometimes with a high density of bacterial cells with a 3D architecture (Figure 1(A)) or spreading along the outer surface of the specimen (Figure 1(B)). As illustrated in Figure 2 using electronic microscopy,

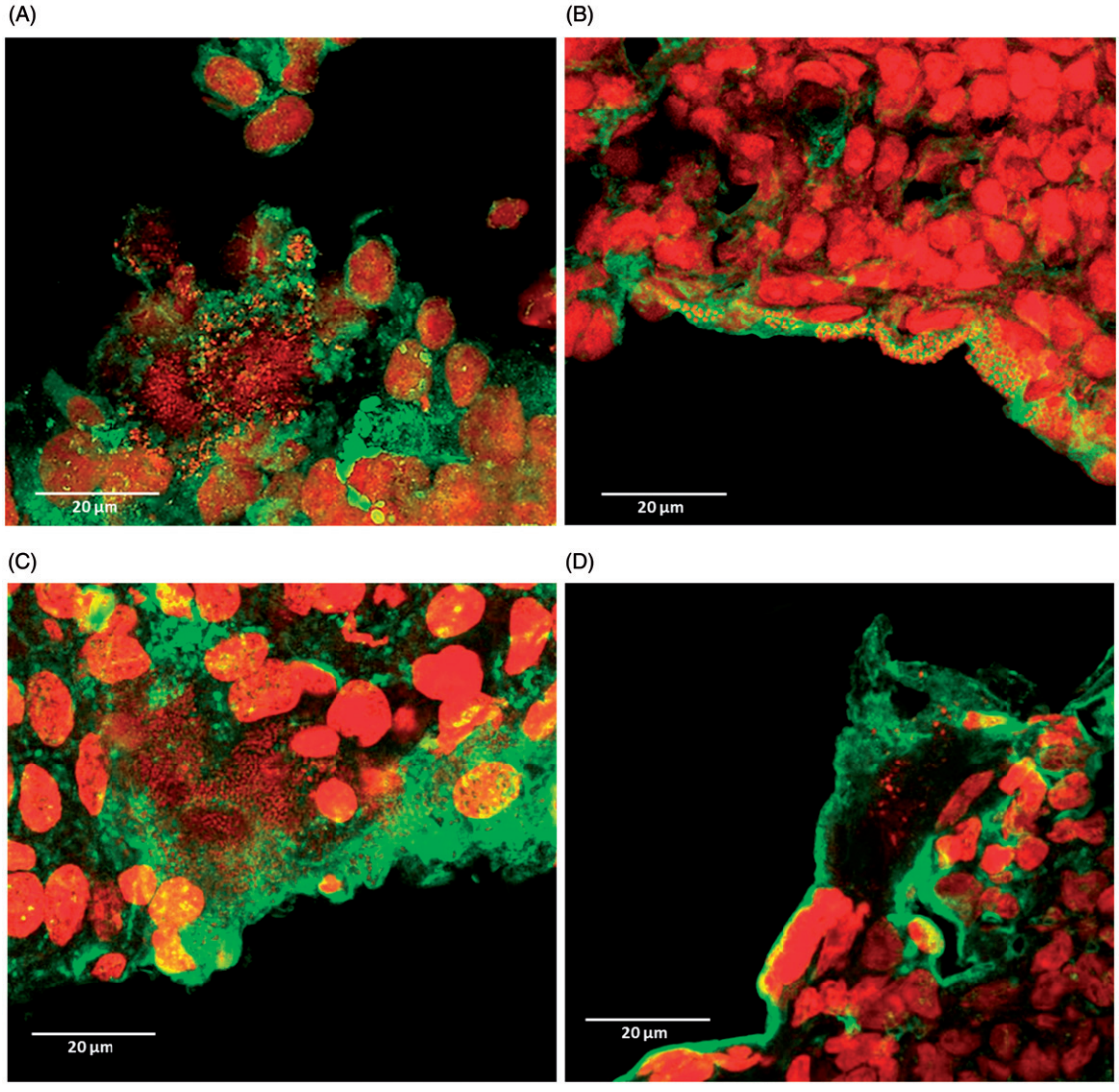

Figure 1. Confocal laser scanning microscopy images demonstrating representative images of bacterial biofilms in adenoids tissue in both groups (A and C group 1 cases of children with COM; B and D group 2, control group i.e children without COM). In A, note the densely packed bacteria in a biofilm building up a 3D architecture. Note in B, how a bacterial biofilm can spread on the surface of the specimen. Note that biofilm can spread into the thickness of the specimen (Figure 1(C)) whereas a biofilm can grow on the surface of the adenoid tissue mucosa (Figure 1(D)).

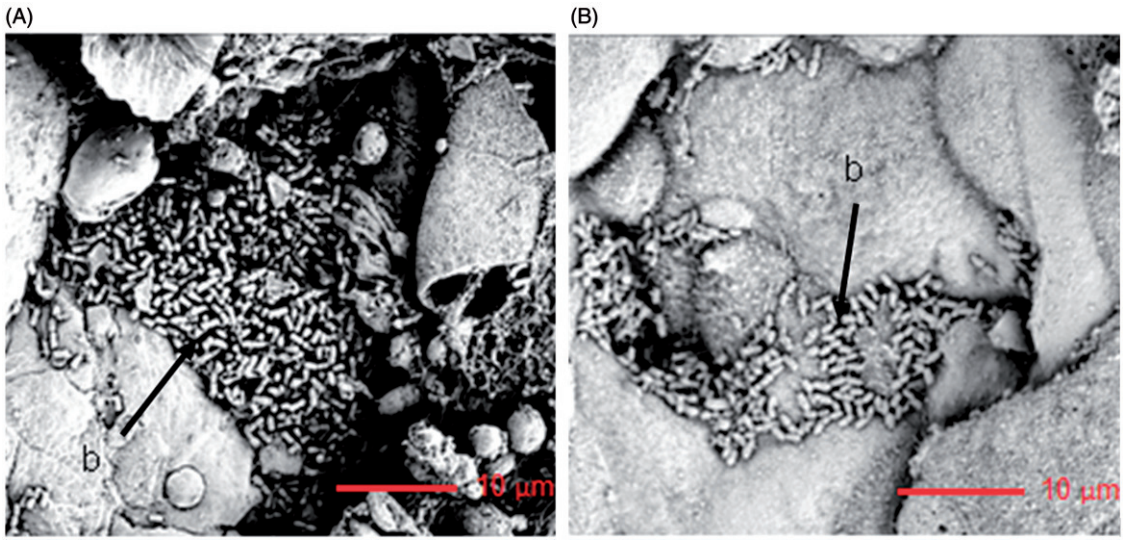

Figure 2. Scanning electron microscopy images showing attached bacteria (b) composing microcolonies connected in some extracellular network in adenoids tissue in both groups (A group 1 cases of children with COM; B, group 2, control group i.e children without COM). In A, note the densely packed bacteria on the surface of the specimen.

Table 1. Variables associated with children with COM (Case group 1) and without COM (Control group 2) to search for risk factors.

Variable	Case (%) ($N = 52$)	Control (%) ($N = 51$)	Odds-ratio (95% CI)	p-value
Breast feed	43 (83)	40 (78)	1.31 (0.49:3.50)	*.58*
Passive smoking	13 (25)	13 (25)	0.97(0.40:2.37)	*.95*
Urban environment	46 (88)	48 (94)	0.48 (0.11:2.03)	*.30*
Young children in siblings	21 (40)	25 (49)	1.42 (0.65:3.10)	*.37*
Day nursery	32 (61)	15 (29)	3.84 (1.69:8.73)	*<.05*
Shared care	4 (8)	4 (8)	0.98 (0.25:3.89)	*.97*
Anemia	1 (2)	7 (14)	0.12 (0.01:1.04)	*.02*
Immune deficiency	1 (2)	0	3.00 (0.12:77.79)	*.31*
Gastroesophageal reflux	13 (25)	7 (13)	2.10 (0.76:5.78)	*.02*
Allergy	7 (13)	11 (22)	*0.57 (0.20:1.60)*	*.28*
Antibiotics	39 (75)	24 (47)	3.37 (1.47:7.77)	*<.05*

interconnected networks of bacteria were found on the outer surface of the specimens in both groups.

The biofilm prevalence was 63.5% (33/52) in group 1 and 47.1% (24/51) in group 2 with odds ratio estimate 1.88 (95% Wald confidence limits 0.85–4.15, $p = .11$). Table 1 summarizes the variables associated in both groups of cases of children with COM (group 1) and without COM (group 2). Day nursery and previous intake of antibiotics three months before adenoidectomy were significantly associated with children with COM. The biofilm prevalence was significantly higher in children with COM (cases group 1) in comparison with the control group 2 with odds ratio estimate 2.87 (95% Wald confidence limits 1.10–7.47, $p = .03$) in a model adjusted to day nursery, young siblings, anemia and antibiotics intake three months before adenoidectomy.

Middle ear samplings

ME biopsies and effusion sampling were obtained in 17 cases of children with COM (group 1). The presence of bacterial biofilms was demonstrated both in the ME mucosa and the ME effusion. As depicted in Figure 3, bacterial biofilms were found both in the ME biopsy specimens and also in ME effusion liquid of children with COM (group 1, cases), with a prevalence of 23.5% (4/17) and 11.8% (2/17) respectively.

Discussion

The main results of the case-control study showed that (i) adenoid tissue in children with COM contained more bacterial biofilms than adenoid tissue removed for hypertrophy, with a prevalence which was significantly higher in children with COM in comparison with the control group. In children with COM, day nursery and antibiotics intake three months before adenoidectomy were significantly more prevalent than in the control group. Bacterial biofilms were found in the ME mucosa and the ME fluid obtained from children with COM. The demonstration of bacterial biofilm was done using CLSM with double-staining providing visualization of bacterial cells and glycocalyx.

Other studies have reported biofilms consisting of identified bacteria or unidentified bacteria on the surfaces of adenoids removed from children with COM, chronic adenotonsillitis, chronic rhinosinusitis and obstructive sleep apnea [5,8–12]. However, our study is, to our knowledge and according to an extensive search in the literature, the largest series including 103 children. Some authors have reported

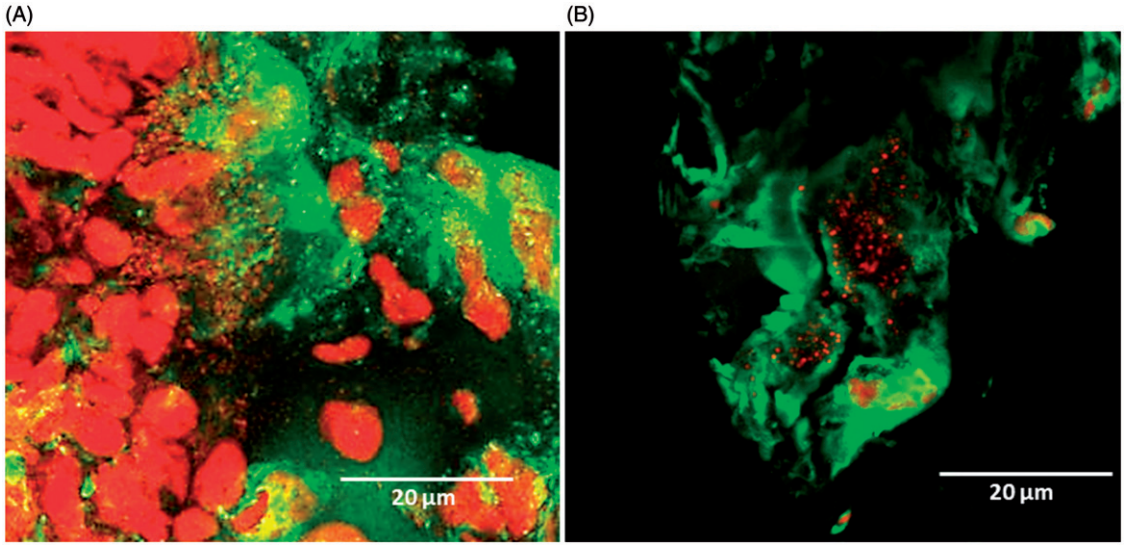

Figure 3. ME biopsy (A) and ME effusion (B) in children with COM (case group 1) showing biofilms on the surface of the ME mucosa (A) and into the lumen of the ME from the effusion sampling (B).

the presence of biofilms in adenoid tissue using SEM [8–12]. Nonetheless, SEM techniques require a dehydration process that reduces the total volume of the matrix and alters its architecture. We have developed a double-staining technique in combination with CLSM which allows simultaneous imaging of the structural elements of a mucosal biofilm [6]. In the present study, this double-staining technique shows bacterial cells and the glycocalyx with the preservation of the architecture of the biofilm due to the lack of dehydration thus providing visualization of bacterial cells and glycocalyx as appearing *in vivo* in their three-dimensional architecture. However, the limitations of our study are: (i) the absence of bacterial identification, (ii) the prevalence of biofilms in adenoids as main outcome measurement without evaluation of the extent of biofilm colonization on the surface of the adenoids, (iii) the absence of quantitative evaluation of adenoids hypertrophy and (iv) an underestimation of the prevalence of biofilms due to the method of search for biofilms on adenoids.

The present case-control study showed that adenoids tissues in both groups contained bacterial biofilms when they were either removed for COM or removed for OSA as hypertrophic adenoids. A higher prevalence of bacterial biofilms was found in children with COM (63.5%) in comparison with the control group with hypertrophic adenoids (47.1%). This observation makes sense because children with COM undergo adenoidectomy for chronic or recurrent bacterial infections, while those with OSA have hypertrophic adenoid tissue not usually accompanying a chronic infection [7]. Further studies are required to identify the bacterial population in the adenoids of children undergoing adenoidectomy for COM and OSA. It can be hypothesized that some bacteria not necessarily highly virulent can be harbored in the adenoids and gain access to the ME space after compromising or risk factors, like viral infection or daycare attendance. In our study, day nursery and previous antibiotics were associated to cases of children with COM, which is not a surprise because it is known that children congregating at daycare promote virus transmission and bacterial reinfection [13]. This may play a role in biofilm production. Similarly, we can assume that cellular stress during inflammatory factors in adenoid tissues may play a role in biofilm production as well. The association between children's care type and otitis media with effusion has been reported [13,14]. The role of antibiotics in biofilm production, in the selection of resistant bacteria and their persistence in a dormant state, has also been reported [3].

Our study demonstrated bacterial biofilms in the ME mucosa and in the ME fluid. Hall-Stoodley et al. first reported in 2006 a direct detection of biofilms visualized by CLSM on 46 (92%) of 50 ME mucosa biopsy specimens from children with otitis media with effusion and recurrent otitis media [15]. The authors suggested that these chronic middle-ear disorders are biofilm-related [15]. Even if the prevalence we herein reported is inferior (23.5%), our study provides supplementary evidence of the existence of bacterial biofilms in the middle ear mucosa. Furthermore, we showed evidence of bacterial biofilm into the lumen of the ME cavity as demonstrated by biofilms found in the ME fluid. One could question the fact that those findings were not surface associated as the concept of biofilm formation is usually built. However, bacteria may form aggregates and biofilms in highly viscous fluid. Actually, biofilms can be associated with neutrophil extracellular traps (NETs) which are formed by activated neutrophils and consist of a DNA backbone embedded with antimicrobial peptides and enzymes [16]. The NETs contribute to the viscosity of the effusion and may negatively impact the clearance of the ME [17,18]. Both fibrin and NETs persist in COM [18]. These structures may provide a scaffold for biofilms building. Therefore, biofilms present in the extremely viscous NETs within the middle ear fluid play a role for the recalcitrance and persistence of middle ear infections [16].

In conclusion, this prospective case-control study demonstrated that more mucosal biofilms were demonstrated in adenoids tissue in children with COM than in adenoid

tissue removed for hypertrophy. Biofilm was seen in the ME mucosa and in the lumen of the ME cavity by sampling the effusion fluid. Even if further studies are required to describe the panel of bacteria that can be harbored in the biofilms present in adenoids and the other mechanisms involved in the physiopathology of otitis-prone children, those observations are in line with the fact that biofilms in adenoids act as a reservoir for COM.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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